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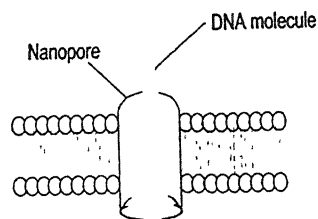
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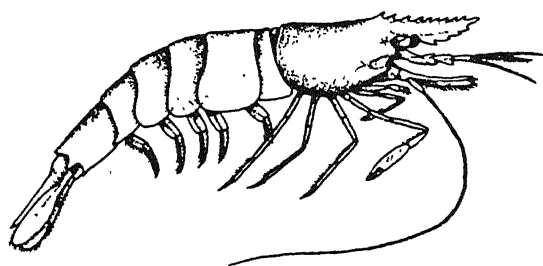
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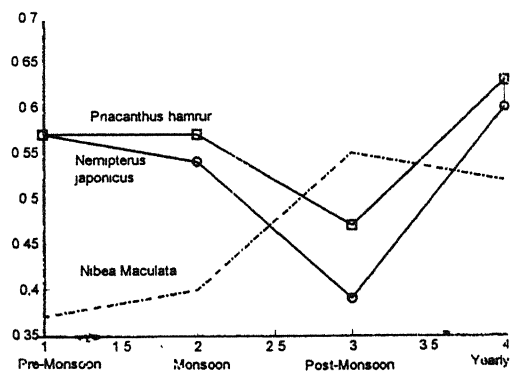
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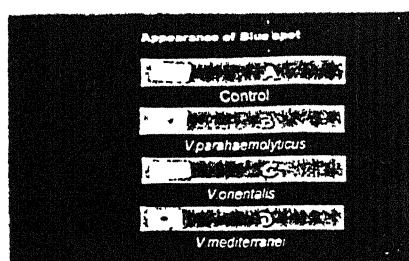
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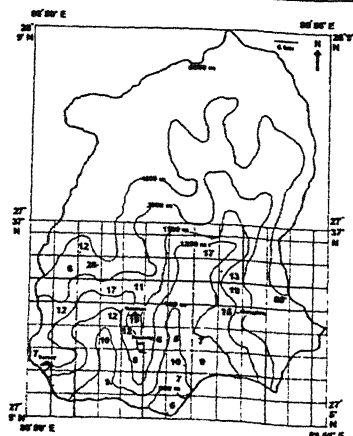
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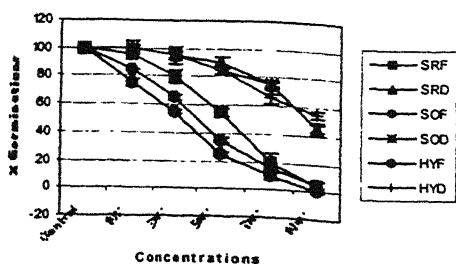
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Detection of trichothecenes and other mycotoxins produced by *Fusarium*

Name of the toxin	Solvent system	Spray reagent	Detection	
			UV	Visible
Deoxynivalenol (DON)	C M (97 3)	4,7,8	-, ch, bl	y,-,-
Diacetoxy scirpenol (DAS)	C M (97 3)	6, 9	bg,-	-, br
Fusarenone - X	C M (97 3)	8	bl	-
HT-2 toxin	C M (97 · 3)	6	bg	-
Moniliformin	C M (97 3)	2, 10	-	br, br
Nivalenol (NIV)	C M (97 3)	4, 7, 8	-, chl, bl	y,-, -
T-2 Toxin	C M (97 3)	6,9	bg,-	-,P
Zearalenone	C M (97 3)	1, 2, 3, 5, 7, 8	-, -, -, br, ch, bl	br, do, lp, -, -, -
Fumonisins (FB1, FB2)	W M (3 · 1)	4,11		br

Solvents C = Chloroform, M = Methanol, W = Water

Natural incidence of fusarial mycotoxins

K. Narasimha Rao, B. Vijayapal Reddy, S. Girisham and S.M. Reddy 164-168

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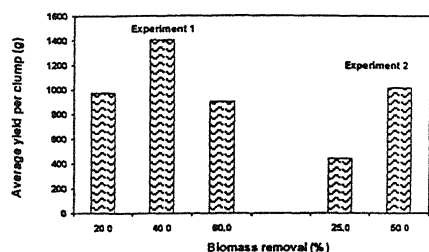
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No of tubers/plant,

Treatment	No of plants/plot		No of tubers/plant (average)	
		2004-05	2005-06	2004-05
Control	, 118	10 64	12 33	32 76
T_1	116	15 43	18 87	74 06
T_2	115	07 52	19 12	24 45
T_3	.12	14 87	24 53	73 76

Environmentally affected allelopathic response of few weeds on late blight disease of potato

S.N. Phukan 174-178

The course of development of late Blight disease of potato was studied in experimental plots planted with seed tubers of cultivar *Khasigaro*, treated with extracts of weed flora namely, *Ludwigia purviflora* Roxb. *Polygonum plabejum* R. Br. and *Anisomeles ovata* Br. The incidence of late Blight was found to be significantly delayed and percent Blight intensity sufficiently decreased when the plants were treated with extracts of *Ludwigia* and *Anisomeles*

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Ultrafast and low-cost DNA sequencing methods for applied genomics research

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Abstract

The availability of human genome sequence and the emergence of personal genomics era recently created a demand to bring down the cost of human genome resequencing to US $ 1000, to make it clinically useful. As a result, sequencing systems using Sanger's technology have been scaled-up, and several ultrafast and low-cost sequencing systems using newer technologies have been developed and launched during 2005-07. Three sequencing platforms, which became available for genomics research in 2007 include the following: (i) Genome Sequencer 20 (launched in 2005) and upgraded Genome Sequencer FLX (launched in 2007), both developed and commercialized jointly by 454 Life Sciences and Roche Applied Science; (ii) Solexa Genome Analyzer (earlier called Solexa 1G) developed by Solexa (later acquired by Illumina) and launched in early 2007, and (iii) SOLiD system, developed and launched in October, 2007 by Applied Biosystems. According to available reports, as many as more than 60 454/Roche Genome Sequencers 20/FLX and at least two dozen Solexa Genome Analyzers are already being used by different genome research centres globally. ABI SOLiD machines will also become available shortly at several centres. Recently. Solexa's sequencing system has also been used for chromatin immunoprecipitation sequencing approach (ChIPSeq) to study DNA methylation and histone modifications at the whole genome level. Several other technologies are also being tested to allow further reduction in cost and time needed for DNA sequencing. For instance, several single molecule sequencing technologies, including nanopore sequencing, which arc likely to become available in foreseeable future, should reduce further the cost and time of DNA sequencing. A major

सारांश

. कुछ समय पहले तक, मानव जीनोम क्रमबद्धता तथा व्यक्तिगत जीनोम के अध्ययन के अविर्भाव ने इस बात की आवश्यकता पर बल दिया है कि मानव जीनोम क्रमबद्धता अध्ययन की लागत घटकर 1000 US $ तक आ जाये तो रोगियो को समुचित लाभ मिल सकेगा। परिणामस्वरूप ऐसे जीनोम को क्रमबद्ध करने की व्यवस्थाये विकसित हो गयी है, जिनमे सेगर की तकनीकी का प्रयोग हुआ हो तथा इन्हे 2005 से 2007 के मध्य बाजार मे उतार दिया गया है। सन् 2007 तक जीनोम से सबद्ध अनुसधान हेतु क्रमबद्ध करने की तीन व्यवस्थाये विकसित हो गई है (i) जीनोम सीक्वेन्स 20 (जो 2005 मे प्रारम्भ हुआ) तथा उन्नत जीनोम सीक्वेन्सर एफ०एल०एक्स० (जो 2007 मे प्रारम्भ हुआ), इन दोनो को एक साथ विकसित करके व्यापारिक व्यवस्था मे 454 लाइफ साइसेज तथा रोश अप्लाइड साइस द्वारा लाया गया। (ii) सोलेक्स जीनोम एनालाइजर (जिसे प्रारभ मे सोलेक्स आई०जी० नाम दिया गया) सोलेक्स (जो बाद मे इल्यूमिना द्वारा अधिग्रहीत कर ली गई) द्वारा विकसित कर के 2007 मे प्रारभ किया गया। (iii) SOLiD तत्र जिसे एप्लाइड बायोसिस्टम द्वारा विकसित करके अक्टूबर 2007 मे प्रारंभ किया गया। प्राप्त सूचनाओं के आधार पर संपूर्ण विश्व के विभिन्न जीनोम अनुसधान केन्द्रो पर 60454 रोश जीनोम सीक्वेन्सर 20/एफ०एल०एक्स० और कम से कम दो दर्जन सोलेक्स जीनोम एनालाइजर उपयोग में लाये जा रहे हैं। कुछ समय पहले से सोलेक्सा सीक्वेन्सिग तत्र का भी प्रयोग संपूर्ण जीनोम के डी०एन०ए० मीथाइलेशन तथा हिस्टोन रूपान्तरण के अध्ययन में क्रोमैटिन इम्यूनोप्रेसिपिटेशन क्रमबद्धता विधि (CHiPSeq) द्वारा किया जा रहा है। डी०एन०ए० की क्रमबद्धता के अध्ययन मे, लागत तथा समय बचाने के उद्देश्य से और भी दूसरी तकनीकी विधिया प्रयोग मे लाई जा रही हैं। उदाहरणार्थ, अनेक एकल परमाणु क्रमबद्धता विधिया जिनमें नैनोपोर क्रमबद्धता विधि भी शामिल है, पूर्वानुमानित भविष्य मे प्रयोग मे लाई जा सकेगी जिससे

lımitatıon wıth some of these modern sequencing systems (e g . Solexa and ABI systems), however, ıs the upper limıt of read lengths of about 35bp per run But, ın parallel with the development of these sequencing systems, computer algorithms are being developed, whıch wıll allow assembly of these small sequences into whole genome sequence, to allow de novo whole genome sequencing even with these machines providıng only short read lengths All these aspects involving a revolution in DNA sequencing technologies are brıefly discussed in this brıef review.

Key words : human genome, genome sequencer 20, solexa genome analyzer, SOLıD system, ChIPSeq, DNA nethylation, hıstone modıfication, nanopore sequencıng.

डी०एन०ए० की क्रमबद्धता के अध्ययन की लागत तथा समय घटाया जा सकेगा। इन आधुनिक क्रमबद्धता के अध्ययन की व्यवस्थाओ मे कुछ एक (सोलेक्सा और सी०बी०आई० व्यवस्था) मे, एक बडा परिसीमन है इनकी "रीड लेन्थ" की ऊपरी सीमा का लगभग 35 बी०पी० प्रति रन का होना। परन्तु इन सीक्वेन्सिग व्यवस्थाओ के विकास के साथ ही साथ कम्प्यूटर एल्गोरिथ्म का भी विकास हो रहा है, जिससे इन छोटे छोटे क्रमो को मिला कर पूरे जीनोम सीक्सेन्स का विस्तृत अध्ययन किया जा सकेगा। इस प्रकार ऐसी मशीनो के द्वारा भी जिनमे केवल छोटे छोटे "रीड लेन्थ" मिलते हो, पूरी जीनोम क्रमबद्धता का अध्ययन किया जा सकता है। प्रस्तुत पुनरावलोकन मे डी०एन०ए० क्रमबद्धता प्रोद्योगिकी क्रान्ति से सबधित सभी प्रारूप वर्णित किये गये है।

सांकेतिक शब्द : मानव जीनोम, जीनोम सीक्वेन्सर 20 सोलेक्सा जीनोम एनालाइजर, SOLıD तत्र, CHIPSeq, डी०एन०ए० मीथाइलेशन, हिस्टोन रूपान्तरण, नैनोपोर क्रमबद्धता।

Introduction

During the current genomics era that started in mid-1990s, whole genome sequencing projects have been completed for a large number of organisms, including several hundred microbes and more than a dozen eukaryotes (including both animal and plant systems). Among these projects, the Human Genome Project (HGP) that was successfully executed and completed in April 2003 was perhaps the biggest biology project (costing US$ 2.7 billion of FY 1991)[1]. The HGP project was compared by some with projects, which involved either the man's landing on moon or the splitting of atom. The completion of these whole genome sequencing projects including HGP created a demand for large-scale high-throughput DNA sequencing, which has never been greater in the past. Although the cost and time for large-scale DNA sequencing is declining steadily, the common sequencing technologies in 2004 were still too expensive and time-consuming to be of practical utility, either in agriculture or in human health-care. Keeping this in view, National Human Genome Research Institute (NHGRI) in 2004, initiated a project entitled "Revolutionary Genome Sequencing Technologies: The $1,000 Genome", aiming at bringing down the cost of whole genome resequencing in humans (for detection of DNA polymorphisms like SNPs) initially to $ 100,000, and eventually to US $ 1000, so that it may become clinically useful for the so-called personal genome project (PGP) during the current era of personal genomics[2] (PGP envisages integration of data from genomes, environments and phenotypes from more than 100,000 human volunteers). Following this announcement, rapid progress has been made in the development of new cost-effective and time-saving DNA sequencing technologies. The published literature and the progress made in this connection have been reviewed in the recent past[3,4], and it has been shown that the progress made actually outpaced earlier expectations[5].

In the early years of the present century, it was recognized that the reduction in the cost and time of sequencing will depend mainly on the following technological developments: (i) miniaturization in instrumentation, (ii) microfluidic separation of substances or molecules, (iii) increase in the number of assays per run. and (iv) development of novel detection methods. These developments sometimes also led to the development of 'lab-on-a-chip' systems (see. Special Issue, Nature Insight, July, 2007). It should be emphasized, however, that we will need new sequencing strategies to make use of these high-throughput platforms effectively. The new strategies leading to the development of platforms for ultrafast low-cost DNA sequencing that have either been developed or are currently under development fall under the following four categories; (i) sequencing-by hybridization (SBH), (ii) sequencing by synthesis (SBS), (iii) sequencing by ligation, and (iv) nanopore sequencing. The present article gives a brief overview of the development of these new DNA sequencing strategies.

Sequencing by hybridization (SBH)

Sequencing by hybridization (SBH) is based on the principle that differential hybridization of oligonucleotide probes, each due to mismatch of a single base, can be used to decode the target DNA sequence. There are at least two alternative approaches for SBH; (i) In the first approach, genomic DNA to be sequenced is first immobilized on a membrane or a glass plate, and is then serially hybridized with short oligonucleotide
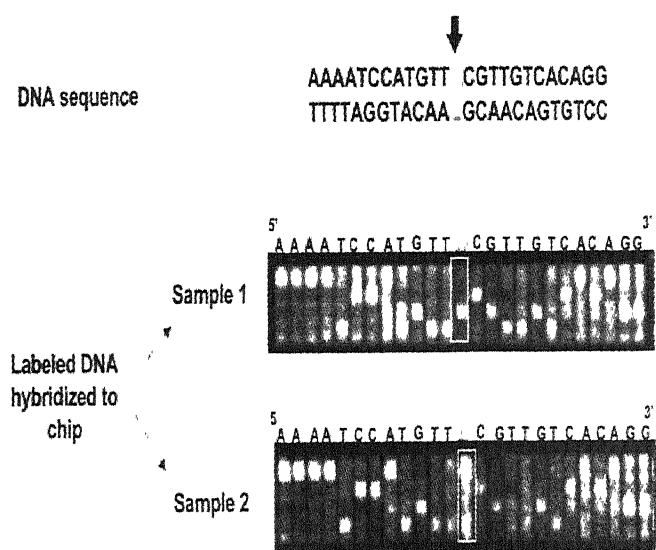
Fig. 1 - A diagrammatic representation of the technique of sequence by hybridization (SBH) using a hypothetical example of single nucleotide polymorphism (modified with permission from Perlegen Sciences) A sequence of 25 base pairs is shown at the top with an arrow at 13th base pair, which is the site of SNP, two panels of hybridized chips show hybridization patterns with two samples, which differ for an SNP at 13th position (GC—> AT).

probes (~7bp) of known sequences. The level of hybridization with each of millions of probes is used to infer the genome sequence. The approach has already been used both for resequencing and de novo sequencing[6,7]. (ii) In the second approach, genomic DNA to be sequenced is hybridized to microfabricated tiling arrays of immobilized oligonucleotides, with ~100,000 copies of each individual oligonucleotide feature (~25bp long) per μm². For each base pair of a genome to be sequenced, there are four features available on the chip corresponding to four possible bases (A, C, G, T). The number of features per base pair will become eight, if both strands are to be examined (as done recently by Perlegen in designing rice oligonucleotide arrays). The strongest signal with only one of the four or with two of the eight features in each case will enable resequencing or sequencing of the whole genome or a part of it. The principle involved in SBH is discussed in more detail elsewhere[8], and is illustrated in Figure 1. The method is more frequently used for detecting single nucleotide polymorphisms (SNPs), and has been successfully used by Perlegen for initially resequencing human chromosome 21[9], and is currently being used by them for resequencing the rice genome for detection of SNPs.

Sequencing by synthesis (SBS)

The SBS technology encompasses many different DNA polymerase-dependent strategies, so that the term SBS has become increasingly ambiguous; therefore, the following classification of DNA polymerase-dependent strategies may prove useful: (a) *sequencing by Sanger's method,* (b) *sequencing by 'single nucleotide addition' (SNA),* (c) *sequencing by 'cyclic reversible termination" (CRT),* (d) FISSEQ polony sequencing, and (e) cyclic array single molecule sequencing..

Sequencing by Sanger's method: current DNA sequencing platforms

Till recently, DNA sequencing techniques involved collection of DNA from an organism (including humans) and then making billions of copies of it to run through sequencing machines. This is slow, expensive, and error prone. This DNA sequencing method (also used for Human Genome Project) was the result of advances in several technologies including the following: (i) enzymology, (ii) fluorescent dyes, (iii) dynamic coating polymers and their derivatives, (iv) capillary array electrophoresis (CAE), and (v) fluorescence detection. In automatic sequence analysers based on these technologies, either the primers or the ddNTPs are labeled with specific fluorescent dyes facilitating detection of fragments resulting from termination of DNA synthesis due to incorporation of ddNTPs.

Improvement over CAE and four colours dye-labeled nucleotides.

The two high-throughput Sanger's method-based CAE automated DNA sequencing machines, which have been extensively used in the past include the following: (i) Applied Biosystems 3730 DNA Analyzer (a 96-capillary DNA sequencer and genotyper); and (ii) the GE MegaBACE DNA Analysis System. The MegaBACE 4000 DNA Analysis system allows sequencing labs to generate up to 2.8 million bases in a 24-hour period by processing 384 samples simultaneously. In recent years, improvements have been made in the above two earlier machines both in respect of technology used for separation of DNA fragments and also in respect of technology used for the detection of separated molecules (see below).

(i) *Microelectrophoretic/microfluidic separation.* Several teams, including researchers at the BioMicroElectroMechanical Systems (BioMEMS) laboratory (located at the MIT Whitehead Institute for

Biomedical Research, Cambridge, Mass.) investigated if costs can be reduced by additional multiplexing and miniaturization of the earlier systems. For this purpose, electrophoresis was performed on microfabricated plates, which represents a very practical, near-term advance using standard Sanger methodology. This led to the production of BioMEMS 768, which was designed specifically for *de novo* long-read sequencing and uses a large format, 384-lane plate (Figure 2). With two of these plates used together, the setup has 768 lanes[10]. In terms of lane count. BioMEMS 768 is eight times the capacity of the Applied Biosystems 3730 DNA Analyzer or twice that of the GE MegaBACE DNA Analysis System.

Although the separation principles of microfabricated devices are similar to those of CAE, but the injection methods differ. For CAE, the sample is introduced by electrokinetic injection into the capillary, but in microfuidic devices, the sample is introduced via a channel network by a variety of process strategies. Thus the time spent in sample injection and in separation of DNA fragments is substantially reduced.

(ii) *Fluorescence detection (FRET and PME technologies).* The most commonly used detection system in sequencing machines that are based on Sanger's method involved four colours, one each for four different nucleotides, which were dye-labeled. These four-colour systems had several disadvantages includ-
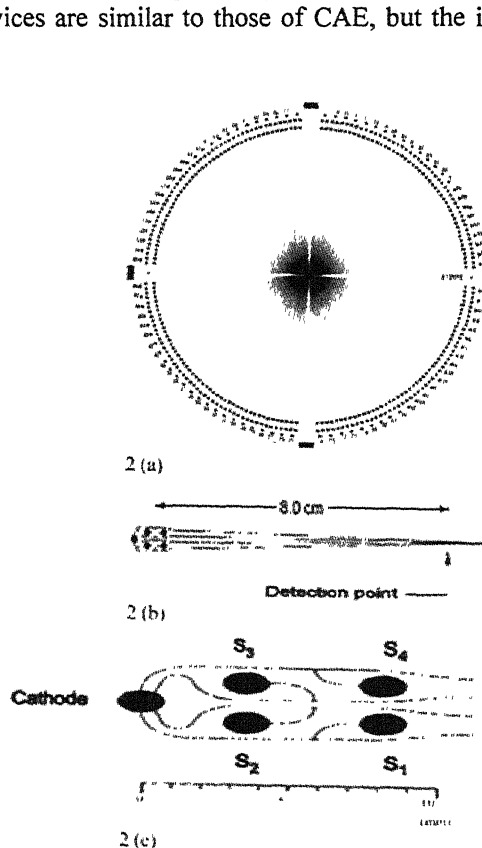
Fig. 2 - A representation of miniaturized microfabricated device for high throughput multiplexing in Sanger's method of DNA sequencing: (a) layout of the 384-lane CAE (capillary array electrophoresis) device on a 200-mm-diameter wafer; (b) expanded view of a single quartet of channels with their injectors; (c) expanded view of one end of a quartet of channels showing a common cathode reservoir located closest to the edge of the wafer (reproduced with permission from Analytical Chemistry, Oct., 2002, 74, page 5077).
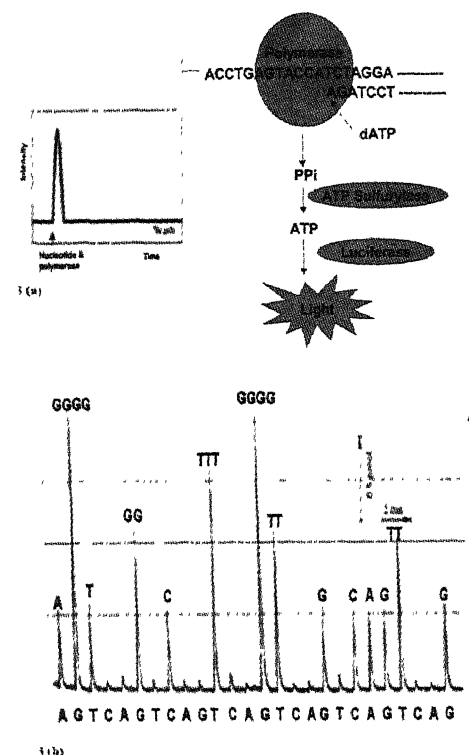
Fig. 3 - Principle involved in DNA sequencing through pyrosequencing used by 454 Life Sciences: (a) schematic representation of the progress of enzyme reaction in solid-phase pyrosequencing. The four different nucleotides are added stepwise to the immobilized primed DNA template and the incorporation event is followed using the enzyme ATP sulfurylase and luciferase. After each nucleotide addition, a washing step is performed to allow iterative addition; (b) a pyrogram of the raw data obtained from liquid-phase pyrosequencing (proportional signals are obtained for one, two, three, and four base- incorporations; nucleotide addition, according to the order of nucleotides, is indicated below the pyrogram and the obtained sequence is indicated above the program).

ing the following: (i) inefficient excitation of fluorescent dyes; (ii) significant spectrum overlap, and (iii) inefficient collection of emission signals. The problem of inefficient excitation was largely overcome through the use of fluorescence resonance energy-transfer (FRET) dyes[11, 12]. FRET-labeled ddNTPs are largely used for DNA sequencing now, although signal intensities are suboptimal, when compared with those of single dyes that are excited at their absorption maxima by the appropriate laser source (see below).

A simple but effective method termed 'pulse multiline excitation' (PME) was also described for multifluorescence discrimination[13]. Advantages of PME include the following: (i) absorption maxima for the four fluorescent dyes are matched to the excitation sources producing maximum signal intensities; (ii) temporal separation of the laser pulses and expansion of the dye set across the visible spectrum eliminate cross-talk between the dyes; and (iii) collection of emission signal is improved by eliminating the requirement for dispersion devices (prism, etc.) for colour separation. In other words, PME measures multicomponent assays in a colour blind manner.

Improvements in the technologies as above have made it possible to sequence one mammalian size genome in one month. This is translated into finishing the draft sequence of a mammalian genome in six months (since six times the genome needs to be sequenced) at a cost of US $ 12 million. Significant advances (discussed later in this article) are needed to bring it to the level of getting complete human genome sequenced within a few hours at an initial cost of $ 100,000, and to an ultimate cost of $ 1000.

Sequencing by single nucleotide addition (SNA): pyrosequencing (454/Roche System)

'Single nucleotide addition (SNA)' methods such as pyrosequencing use limiting amounts of individual natural dNTPs to cause DNA synthesis to pause (whenever the limiting nucleotide is required for extension). Unlike Sanger's method, synthesis can be resumed with the addition of the complementary nucleotide. A sequential addition of dNTPs and the generation of chemiluminescence signal (through luciferin) that is proportional to the number of molecules of the same base added, is used to get the sequence (Figure 3). The amount of a given dNTP needs to be limited to minimize misincorporation effects observed at higher concentrations. A major drawback with this SNA approach is the incomplete extension through homopolymer repeats.

An emulsion method for DNA amplification and an instrument for sequencing by synthesis using a pyrosequencing protocol involving solid support and picolitre-scale volumes was initially optimized by 454 Life Sciences (Branford, Connecticut). This gave 100-fold increase in throughput over current Sanger sequencing technology[14]. Later, 454 Life Sciences and Roche Applied Science jointly commercialized sequencing systems based on emulsion method of single molecule DNA amplification and pyrosequencing. In this system called 'Genome Sequencer 20 System', no gels or fluorescent molecules are needed and hundreds of thousands of DNA fragments can be analysed in parallel, making it suitable for whole genome sequencing or genome-wide SNP genotyping. An upgraded Genome Sequencer FLX system, later launched, allows more sequencing cycles and therefore, longer reads than the Genome Sequencer 20 earlier launched. The 454/Roche pyrosequencing technology has already been successfully used for sequencing of the genome of a Neanderthal reported in 2003[15], and that of the genome of James Watson reported in May, 2007. In October 2007, as many as 60 454/Roche sequencing systems were already being used at Genome centres world-wide[2], and further improvement in these machines are being tried.

Following steps are involved in the technology used in 454/Roche Sequencing System;

(i) DNA library preparation: A single-stranded DNA (sstDNA) library with DNA fragments incorporating primer binding adaptors is first prepared using a series of enzymatic steps. For this purpose, genomic DNA (3μg) is first fragmented by nebulization into smaller fragments (300-800bpJ, which are polished (blunted) before short adaptors A and B are ligated, and a streptavidin binding site is created in each fragment for sample purification. Single DNA molecules are isolated by limiting dilution. The sstDNA library produced thus is assessed for quality and subjected to titration for finding out the optimal amount of DNA (copies per bead) needed for emPCR (em = emulsified).

(ii) emPCR amplification. The sst library prepared as above is immobilized onto specially designed DNA Capture Beads, each bead carrying a single fragment. The bead-bound library is emulsified with amplification reagents in a water-in-oil mixture. Each bead is separately captured within its own microreactor

for PCR amplification, which is performed in bulk thus producing clonally amplified library on beads, each bead carrying millions of copies of a specific fragment to be used for sequencing. Individual fragments on their own beads are clonally amplified within the droplets of an emulsion.

(iii) Preparation of PicoTitre Plate device. After PCR amplification, a combination of amplified sst library on beads and sequencing enzymes are loaded onto hundreds of thousands of wells available in PicoTitre Plate device, which is then placed into the instrument for sequencing-by-synthesis (GS20/FLX Sequencing System).

(iv) Sequencing-by-synthesis. Sequencing starts with a flow of sequencing reagents (buffers + nucleotides) across wells of the PicoTtitre Plate. Each sequencing cycle involves flowing of individual nucleotides in a fixed order (e.g., T, A, C, G), so that if the nucleotide is complementary to the available base in the template, the strand is extended releasing a pyrophosphate molecule, which produces a chemiluminescent signal with luciferin (signal strength is proportional to the number of nucleotides incorporated in a single flow). Instead, if the supplied base is not complementary, there is a pause, and no signal is produced. These cycles of SNA proceed concurrently in all wells of the PicoTtitre Plate, which permits DNA sequencing of hundreds of thousands of DNA fragments in parallel.

Sequencing by 'cyclic reversible termination' (CRT): Solexa technology

This CRT method used in Solexa technology makes use of nucleotides that are reversibly fluorescent-labeled and also protected against any condensation reaction for further SNA, unless deprotected again. Consequently, after each single nucleotide addition, no further extension is possible unless the terminal nucleotide is first deprotected. The fluorescent label is also erased, so that it does not interfere with the detection of the next nucleotide to be added. Thus each cycle of SNA involves addition of single nucleotide, its detection using a sensitive device, erasure of fluorescence and deprotection of the terminal nucleotide, followed by addition of the next reversibly labeled and protected nucleotide (Figure 4). Solexa has standardized this technology and has developed a sequencing system that was marketed in 2007. The system developed by Solexa allows a single person to produce up to 40 megabases of sequencing in a 4.5 hour run. Sequencing a new organism thus takes approximately one week from start to finish, with repeat runs taking as little as one day to complete. During 2006, Solexa demonstrated an yield of 1G (1G = 1 gigabase = 10^9 bases) high-quality DNA sequence data per flow cell after filtering, using multiple runs on multiple instruments, making Solexa Genome Analysis System the first to achieve this milestone. Solexa Sequencing System was initially called 'Solexa 1G', but later named 'Genome Analyzer'. Solexa's Genome Analyzer has already been used for the study of the patterns of chromatin structure through chromatin immunoprecipitation sequencing (ChIPSeq) technique, and will certainly be used in future for transcription profiling, microRNA/small RNA discovery, and to identify polymorphisms in closely related genomes (for ChIPSeq, see later in this article). Further improvements will be made in the technology during 2008 and thereafter, so that it will be able to achieve the targeted goal of $1,000 human genome sequencing within the next 2-3 years.

Following steps are involved in the above CRT technology of Solexa :

(i) Preparation of full diversity libraries of whole genome: Whole genome DNA is randomly fragmented and end repaired. Solexa oligonucleotide adapters are ligated on both the ends of each of these fragments. This gives a fully representative genomic library of DNA templates without cloning.
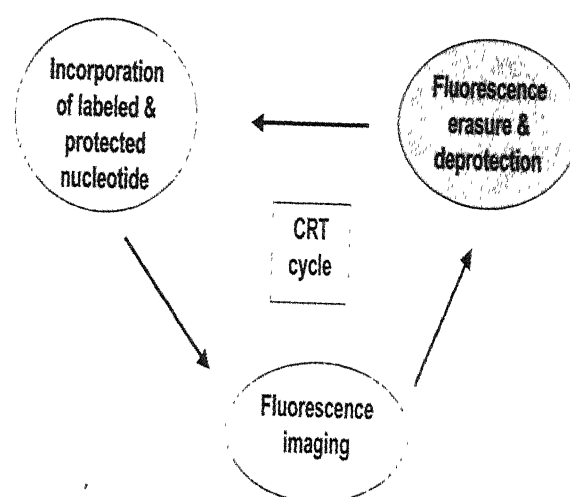
Fig. 4 - A diagrammatic representation of three major events involved in cyclic reverse termination (CRT) during DNA sequencing approach utilized by Solexa.

(ii) Generating a "Clonal Single Molecule Array™" Flow Cell: Single molecules from the genomic library are amplified, which involves following six steps: (i) template hybridization; (ii) template amplification; (iii) linearization; (iv) blocking 3' ends; (v) denaturation, and (vi) primer hybridization. These steps are performed on a fully automated Solexa Cluster Station, creating up to 1000 copies of each single molecule, and giving a density of up to 10 million clonal clusters per cm^2.

(iii) Sequencing by synthesis. The flow cell with clonal clusters (each cluster representing only one molecule) is loaded onto the 'Solexa 1G Genetic Analyzer' (later named 'Genome Analyzer'), which conducts automated sequencing by synthesis (SBS). The SBS utilizes four proprietary nucleotides with reversible fluorophores and termination properties. Each sequencing cycle occurs in the presence of ail four nucleotides leading to higher accuracy. After incorporation of a nucleotide, reaction terminates, the fluorescence is read and then erased, the terminal nucleoiide is deprotected and the new synthesis cycle starts. At each replication cycle, the data is automatically recorded from each cluster, processed and analysed with minimum intervention by the user. This stepwise base addition approach, which cycles between coupling and deprotection, mimics many of the steps involved in automated DNA synthesizers used for synthesis of oligonucleotides.

Fluorescent in situ sequencing (FISSEQ) or bead-based polony sequencing

FISSEQ involves localized amplification of single DNA molecules, using an acrylamide gel, thus creating colonies of PCR product that are called polonies. Because acrylamide restricts the diffusion of DNA, each single molecule included in the reaction produces a spatially distinct micrometer-scale colony of DNA (a polony), which can be sequenced *in situ* independently of similar other polonies (Figure 5).

An extension of the original FISSEQ technique, termed 'bead-based polony sequencing', was developed by Jay Shendure and colleagues in George Church's Lab at Harvard Medical School, Boston, and has been licensed to Agencourt Biosciences, Beverly, Mass. In this massively parallel sequencing-by-synthesis approach, short fragment DNA libraries are clonally amplified onto 1-µm beads and embedded into a polymer matrix on the surface of microscope slides. The polony slides are then placed into an automated flow cell, where four-
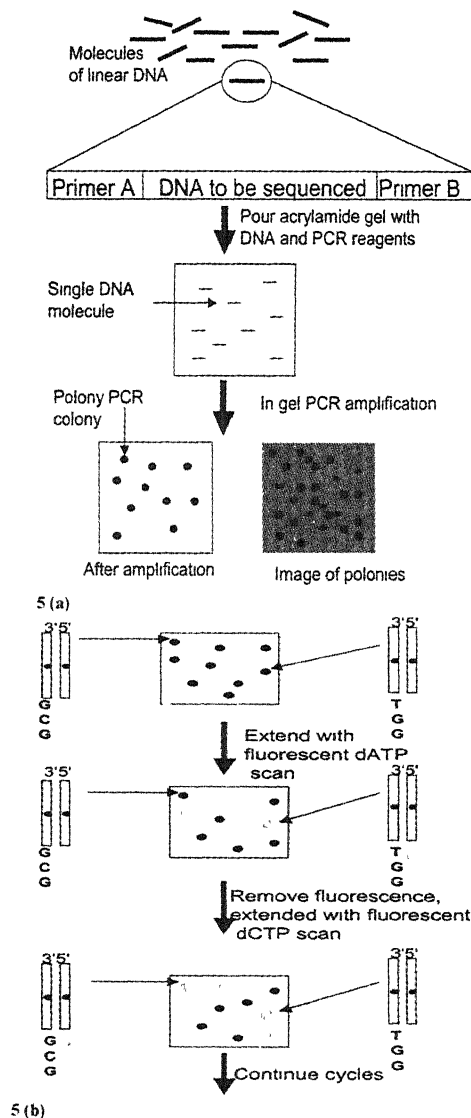
Fig. 5 - (a) Polony amplification. A library of linear DNA molecules with universal priming sites is PCR amplified in a polyacrylamide gel. A single template molecule gives rise to a polymerase colony or polony, (b) Fluorescent in situ sequencing. Polonies are denatured, and a sequencing primer is annealed. Polonies are sequenced by serial additions of a single fluorescent nucleotide.

The sequence GCG shown on the right belongs to the upper right polony and the sequence TGG shown on the left belongs to middle left polony (both sequences shown along with primers and corresponding polonies are marked with arrows). When fluorescent labeled dATP is added in the first round of sequencing reaction, no primer extension is possible in the polony on the right, but extension is allowed in polony on the left; similarly, when dCTP is added in the second round, primer extension takes place in both polonies, thus permitting detection of sequence polymorphism.

colour, fluorescently labeled reagents (corresponding to the DNA bases, A, C, G, or T) are serially delivered to DNA strands. Each polony slide can contain up to 600 million beads with the instrument capable of sequencing 100,000 bases per second, equivalent to approximately 5,000 Applied Biosystems (ABI) 3730 DNA analyzers. It is hoped that the technology will prove useful for personalized medicine and for low-cost genome analysis.

Cyclic array single molecule sequencing

In each of the SNA methods described above, separated single DNA molecules are first amplified, before these are used for DNA sequencing, so that the required signal is achieved. However, if single molecules could be sequenced directly without the need of amplification, this would save cost and time needed for cloning/ amplification. Several groups including Solexa, Genovoxx, Nanofluidics and Helicos are developing cyclic array methods, which though make use of technologies used for other DNA sequencing methods discussed above, but dispense with the amplification of single molecules. Each of these methods makes use of either the excision of labeled mononucleotides using exonuclease ! or the extension of DNA fragments by DNA polymerase using fluorescently labeled nucleotides[16]. The methods, however, differ in biochemistry and signal detection devices at a resolution of single molecules.

Common approaches for the study of single molecules include fluorescence correlation spectroscopy (PCS) and direct observation of sparse molecules using diffraction-limited optics. These approaches provide observation volumes on the order of 0.20 fl (fl = femtolitre; 1 fl = 10^{-15} litre) and therefore require pico- to nanomolar concentrations of fluorophores in order to isolate individual molecules in solution. However, many biological processes occur at micromolar concentration (rather than nano- or picomolar concentration), so that observation volume has to be reduced by three orders of magnitude to zeptolitre (1 zeptolitre = 10^{-21} litre) in order to isolate single molecules at these higher micromolar concentrations. Zero-mode waveguides consisting of tiny holes (subwavelngth diameter = nanopores) in a metal film have been used for studying single molecule dynamics at micromolar concentrations with microsecond temporal resolution[17]. Following are some of the methods, where sequencing is achieved using single molecules.

(i) Reversible terminators. As discussed earlier, reversible terminators that are being used by Solexa/ Illumina sequencing systems, are also being tried for single molecule sequencing by Solexa and Genovoxx, except that no amplification step will be involved.

(ii) FRET for serial single base extension. Quake group makes use of single DNA molecules for serial single base extension, which are detected using FRET to improve signal-to-noise ratio.

(iii) DNA sequencing using RNA polymerase. Recently, a sequencing method was proposed that involved resolution of the motion of a processive RNA polymerase enzyme. This method involves tracking of transcription by single molecules of RNA polymerase (RNAP). A pair of optical traps levitates two polystyrene beads, one attached to RNAP and other to the distal end of the template being sequenced. Motion of RNAP during transcription changes the length of the DNA segment joining the two beads, which can be recorded with precision, providing single base-pair resolution. When transcription is carried out with one of the four nucleotides (NTPs) at a very low concentration, RNAP will pause if it needs the limiting nucleotide. In this manner if four transcription reactions are set up, each with a different rate limiting NTP, RNAP will pause in three of the four reactions, and this will be recorded as time, The records of four reactions can then be aligned to obtain the DNA sequence. The success of this method has recently been demonstrated[18].

Sequencing by ligation (SBL)

A new generation DNA sequencing system, employing a novel approach (sequencing by ligation or SBL), earlier developed by Agencourt Personal Genomics (APG), is also being launched by Applied Biosystems, who has acquired APG. The approach is based on what has been described as Supported Oligo Ligation Detection (SOLiD™) process and may compete with the following two new generation DNA sequencing systems already launched and earlier described in this article: (i) the Roche Diagnostics/454 Life Sciences Genome Sequencer FLX system, and (ii) the Illumina/Solexa Genome Analyser.

SOLiD™ from Applied Biosystems

The above SOLiD™ of Applied Biosystems involves the following five steps: (i) *sample preparation,* which
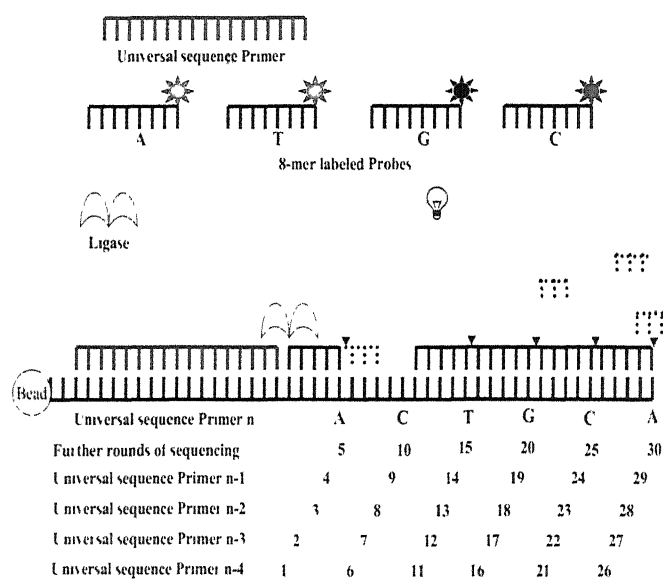
Fig 6 - A representation of the strategy used in Supported Oligo Ligation Detection (SOLiD^{TM}) process that is used in DNA sequencing system launched by Applied Biosystems. On the top are shown (i) a 17-mer universal primer that pairs with adapter attached to each fragment to be sequenced (these adapter ligated fragments are each attached to a microbead and em-PCR amplified), (ii) four 8-mer probes differing from each other only at the 5th interrogating base The universal primer is ligated with each of the four probes separately, and the four primer-probe fragments are annealed serially in order with each adapter-ligated and -amplified fragment. After each round of annealing, the probe is cleaved at the 5th base, to allow annealing of another primer probe fragment. The fluorescent dyes allow identification (base-calling) of bases on positions 5th, 10th, 15th, 20th in order In subsequent rounds the primers of length n-1, n-2, n-3, n-4, are used, so that bases at positions 4, 9, 14, 19 in the second round, bases at positions 3, 8, 13, 18 in the third round, bases at positions 2, 7, 12, 17 in the fourth round and bases at positions 1, 6, 11, 16 in the fifth round are identified. This will thus give the complete sequence of the fragment in five rounds of annealing and base-calling.

involves development of a genomic library with DNA fragments (~2kb), each with adapters of known sequence ligated at its ends to facilitate designing primers for PCR reactions; (ii) *em-PCR and substrate preparation,* which involves amplification of solitary DNA fragments on individual microbeads using emulsified DNA; (iii) *ligation reaction,* which involves ligation of universal sequencing primer with each of the four 8-base long oligo probes (the fifth base being an interrogating base), each labeled with a fluorescent dye; the ligated primer-probe oligos bind with the adapter-ligated template DNA (probes are used in a set of four oligos, which differ only at the fifth position; the four oligos are distinguished by four different fluorescent dyes used for labeling); (iv) *imaging and base-calling,* which involves imaging facilitated by the fluorescent dye label that identifies the fifth interrogating base (an activity described as base-calling); the cycle involving ligation, imaging/base-calling and cleavage continues and allows identification of bases at positions 5, 10, 15, 20, 25.........5n (Figure 6). The intervening bases (1-4; 6-9; 11-14; 16-19; 21-24, etc.) are identified through sequential rounds of sequencing using four additional primers with lengths n-1, n-2, n-3 and n-4. The process allows simultaneous reading of millions of DNA fragments in a massively parallel manner.

Nanopore sequencing

Back in 1989, UCSC Professor David Deamer first conceived of making nanopores through which a single strand of DNA would pass at a time and as the strand passed through the nanopore, its changing electrical pattern would be used to read each successive DNA base via sensors built into the nanopore structure (Figure 7). This approach holds the potential of allowing for miniaturization, elimination of lots of expensive reagents, and to speed sequencing by many orders of magnitude. The detection schemes being developed will transduce the different chemical and physical properties of each base into a characteristic electronic signal. Nanopore sequencing has the potential of reading very long stretches of DNA at rates exceeding 1 base per millisecond

The physicists used mathematical calculations and computer modeling of the motions and electrical fluctuations of DNA molecules to determine how to distinguish each of the four different bases (A, G, C, T) that constitute a strand of DNA. They based their calculations on a pore about a nanometer in diameter made from silicon nitride-a material that is easy to work with and commonly used in nanostructures-surrounded by two pairs of tiny gold electrodes. The electrodes would record the electrical current perpendicular to the DNA strand as the DNA passed through the pore. Because each DNA base is structurally and chemically different, each base creates its own distinct electronic signature. The nanopore DNA sequencing method has the potential of having fewer errors than other methods. It should be possible to sequence strands of DNA that are tens of thousands of base pairs in length (possibly as long as an entire gene), in one pass through the nanopore.
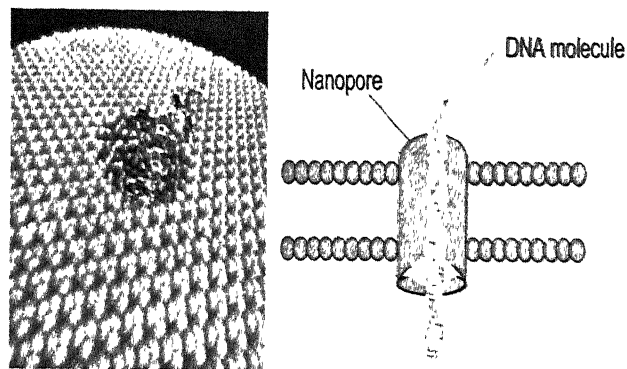
Fig. 7 - The device and the approach proposed in nanopore sequencing that is likely to be used in future. (a) surface view of a nanopore sequencing device; (b) a single DNA molecule travels through the pore; the chemical and physical properties of each base are converted into a characteristic electronic signal to be read by the electrodes fitted in the nanopore (http://ucsdnews.ucsd.edu/newsrel/science/sfastdna asp).

Previous attempts to sequence DNA using nanopores were not successful because the twisting and turning of the DNA strand introduced too much noise into the signal being recorded. The new idea takes advantage of the electric field that drives the current perpendicular to the DNA strand to reduce the structural fluctuations of DNA, while it moves through the pore, thus minimizing the noise. The researchers, however, caution that there are still hurdles to overcome because no one has yet made a nanopore with the required configuration of electrodes, but they think it is only a matter of time before someone successfully assembles the device. The nanopore and the electrodes have been made separately, and although it is technically challenging to bring them together, the field is advancing so rapidly that they think it should be possible in the near future.

ChIPSeq for genome-wide maps of protein binding and histone modification sites (one genome with several epigenomes)

The high-throughput low-cost DNA sequencing platforms discussed above are also being used for a variety of other studies. One such application is the study of genome-wide interactions between a specific protein and genomic DNA, or else the study of the epigenome in different cell/tissue types, each type exhibiting a specific histone modification at specific sites of genomic DNA. In either case, ChIPSeq technique has been used, in which an antibody raised against the protein or the modified histone of interest is used to selectively coimmunoprecipitate the DNA associated with the protein/histone of interest. The DNA is recovered from DNA-protein complexes and is used for high-throughput DNA sequencing to allow identification of DNA sites associated with specific protein or the modified histone[19]. The technique has been successfully used for identification of 1946 locations in human genome for binding of transcription factor NRSF (neuron-restrictive silencer factor)[20]. Similarly, in another study, 41,582 and 11,004 putative STAT1-binding regions were identified in stimulated and unstimulated human cells respectively[21]. In both these studies, Solexa/Illumina 1G machine was used for high-throughput sequencing of millions of small DNA fragments of~30 bases length that were ideal for characterizing ChIP derived fragments.

In July/August, 2007, two additional reports were published, where Solexa 1G machine was used for construction of whole-genome maps of chromatin states for a variety of mouse and human cells involving different histone modifications (methylation of different lysine and arginine residues of H3 histone). In one of these studies, sequences of four billion bases of immunoprecipitated DNA were used to generate genome-wide chromatin-state maps of mouse embryonic stem cells, neural progenitor cells and embryonic fibroblasts[22]. H3K27me3 was found to be an important mark that discriminated between genes that are expressed or poised to express and those which are repressed. Using single nucleotide polymorphisms (SNPs), the chromatin states could also be read in an allele-specific manner. Similarly, in the other study of human epigenomes, high-resolution maps of the distribution of 20 histone lysine and arginine methylations as well as histone variant H2A.Z were generated[23]. Typical patterns of histone methylations were identified at promoters, insulators, enhancers, and transcribed regions; monomethylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 were associated with gene activation, while trimethylations of H3K27, H3K9, and H3K79 were linked to repression.

A comparison of three new generation sequencing systems

A comparison of three sequencing systems that became available in 2007 is presented in Table 1, which shows superiority of Solexa and ABI SOLiD over 454

Genome Sequencer FLX in terms of time, cost, precision, and ease of using, However, the only disadvantage with these systems is the read length, which is only 25-36 bases, which makes it relatively unsuitable for de novo whole genome sequencing, and renders them suitable for only resequencing, transcript discovery and for whole genome ChIPSeq. Therefore, questions have been raised whether; de novo accurate assembly can be achieved with sequences as small as only 25-26 bp long at an acceptable computational cost.

We know that for the assembly of sequence fragments of ~500 bp in length, a variety of sophisticated assembly algorithms have been available in the past and are still being used, but short-read assembly algorithms for de novo whole genome sequencing were not available. As the demand for such algorithms increased with the availability of new generation of Solexa and ABI SOLiD sequencing systems, two recent papers reported availability of algorithms SSAKE[24] and SHARCGS[25], which are claimed to facilitate assembly of sequences that are 25-36 bp long. Since SSAKE was found to allow some misassemblies, SHARCGS was designed to overcome this problem. With the availability of these algorithms, the main hurdle of using Solexa and ABI SOLiD for de novo whole genome sequencing has largely been overcome. With the availability of these softwares and others to be developed, the new generation sequencing systems will certainly replace the Sanger sequencing technology for even the de novo whole genome sequencing (WGS).

Table 1 - A comparison of three new generation DNA sequencing platforms

Features	454 FLX	Solexa	ABI SOLiD
Read length	250bp(350)	25-36bp (50)	25bp (30)
Number of reads/run	420K	~40M	40M
Data output	~100Mb	~1Gb	~3Gb
Cost per run	~$8,000	~$3,000	~$6,000
Cost per Gb	~$80.000	~$3,000	~$3,000
Time per run	8 hours	3 days	3 days
Run time to 1Gb	10 days	2-3 days	7- 10 days
Ease of use	Most difficult	Least difficult	Difficult
Error	0.5%	0.2%	0.1%

Conclusions

The development of low-cost ultrafast DNA sequencing methods has witnessed a revolution in the recent past. At least three new generation sequencing systems have already been launched, one each by 454 Life Sciences/Roche (based on pyrosequencing), Solexa/Illumina (based on cyclic reverse termination) and ABI or Applied Biosystems (based on sequencing by ligation or SOLiD™). Solexa Genome Analyzer can generate 1 Gb and ABI SOLiD can generate 2-4 Gb sequence in one single run. The 454/Roche system, which is said to be half as fast has also been utilized to sequence the genome of James Watson in the early months of 2007. Several other companies are developing even faster technologies that are not being publicly discussed. Among these, several single-molecule sequencing methods that are being examined for their use in DNA sequencing rely on measurement of either the motion of a DNA/RNA polymerase, or the current across single DNA molecule traversing through a nanopore, or the fluorescence emitted from an individual nucleotide. These, methods, however, are in early stages of development, and several problems associated with them need to be overcome before these can be utilized for actual DNA sequencing for genomics research. If not in the near future, some of these single-molecule sequencing methods should also become available in the long-term.

The above developments have already brought down (or are likely to bring down by 2010) the cost of whole human genome sequencing to $ 100,000. but bringing it down to the targeted $ 1000 still remains a challenge. If the sequencing of human genome is brought down to the targeted $ 1000, this will translate to sequencing a maize genome at an equivalent cost of $ 1000 and sequencing a rice genome at a cost of $ 135, thus making it feasible to utilize this technology on a large-scale for applied plant genomics also. In any case, it is certain that the state-of-the-art of DNA sequencing technology will dramatically change within the next 2-5 years, thus bringing about a revolution in applied genomics research.

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Major diseases and the defence mechanism in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man)

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Abstract

The giant freshwater prawn, *Macrobrachium rosenbergu* de Man or scampi is an economically important farmed crustacean species all over the world. India contributes ~ 20% to the world's scampi aquaculture production, being ranked second in the world In recent years, the rapid expansion and intensification of culture practices have brought several diseases of infectious and noninfectious aetiologies. The increased globalization of trade and transboundry movement of broodstock and postlarvae, the unanticipated interactions between cultured and wild populations, poor biosecurity measures, lack of awareness on emerging pathogens, climate change, misuse of drugs and antibiotics and other human-mediated movement of aquaculture produce and products are playing key role in occurrence, spread and outbreaks of diseases. The main causative agents of the infectious diseases are viruses, bacteria, rickettsia, fungi and protozoa. In past few years, nodavirus is causing a devastating production loss to scampi industry in many countries including India. So disease control is becoming a priority. The defence mechanism of giant freshwater prawn, *M rosenbergu* de Man. is poorly understood The knowledge of the functioning of its defence system is of extreme importance. The stimulation of this system is considered as a potential intervention strategy in scampi culture to overcome the infectious diseases. This review focuses on the recent information of major diseases of giant freshwater prawn, *M rosenbergu* de Man. and the related defence mechanism which may be of help for sustainable development of fast-growing scampi industry.

Key words : *Macrobrachium rosenbergii*, major diseases, defence mechanism

सारांश

मीठाजल महाकायझीगा, *मैक्रोब्रैकियम रोजेनबर्गाई* या स्कैम्पी सपूर्ण विश्व मे आर्थिक रूप से एक महत्वपूर्ण क्रस्टेशियन पालन प्रजाति है। सपूर्ण विश्व के कुल स्कैम्पी उत्पादन मे भारत ~ 20% की भागीदारी करते हुये द्वितीय स्थान पर आता है। हाल के वर्षों मे लगातार बढते हुये पालन क्षेत्र एव सघनीकरण के कारण इनमे अनेक तरह के सक्रामक एव असक्रामक रोग भी प्रकट हो रहे है। इसके अतिरिक्त बढता हुआ विश्व व्यापार, प्रजनको एव पोस्टलार्वा का देश की सीमाओ से बाहर पालायन, पालतू एव जगली प्रजातियो का अप्रत्याशित मिलन, कमजोर जैव सुरक्षा, प्रकट होने वाले रोगाणुओ के बारे मे अनभिज्ञता, जलवायु परिवर्तन, औषधियो एव एन्टीबायोटिक का दुरूपयोग, एक्वाकल्चर, उत्पाद एव उत्पादन का मानव द्वारा परिगमन, विभिन्न रोगो की उत्पत्ति, इनके फैलने तथा वृहद रूप धारण करने मे महत्वपूर्ण भूमिका ऩिभा रहे हैं। सक्रामक रोग प्रमुखत वाइरस, बैक्टीरिया, रिकेट्सिया, कवक तथा प्रोटोज़ोआ द्वारा उत्पन्न होते है। भारत सहित विश्व के अनेक देशो मे नोडावाइरस के कारण पिछले कुछ वर्षो मे स्कैम्पी उत्पादन मे अत्यधिक क्षति हुई है। अत बीमारियो की रोकथाम करना एक प्राथमिकता होती जा रही है। मीठेजल के महाझीगा *मैक्रोबैकियम रोजेनबर्गाई* की प्रतिरोधी व्यवस्था का ज्ञान अत्यधिक सकुचित है, परन्तु यह अत्यधिक महत्वपूर्ण है। इस तत्र के उद्दीपन मात्र से स्कैम्पी के सक्रमक रोगो से बचाव सभावित है। प्रस्तुत आलेख मे, मीठाजल महाझीगा के प्रमुख रोगो से सबद्ध नवीनतम सूचनाये तथा इनसे सबधित प्रतिरोधी व्यवस्थाये वर्णित है, जो त्वरित रूप मे बढते स्कैम्पी उद्यम के सतत् विकास मे सहायक हो सकती है।

सांकेतिक शब्द : *मैक्रोब्रैकियम :रोजेनबर्गाई*, प्रमुख रोग, प्रतिरोधक तत्र।

Introduction

Aquaculture plays a major role in increasing fish and shellfish production in tropical countries like India. The vast aquatic resources and conducive environment of India favour rapid expansion of freshwater prawn farming activities. The giant freshwater prawn, *M. rosenbergii*, popularly known as scampi, is the largest and fastest growing freshwater prawn species. It has received considerable attention in recent years due to higher profit realization per unit area[1, 2, 3]. The ever-increasing demand of the species in

the international market further provides enormous scope for expansion of its cultivation. About four million ha of impounded freshwater bodies in various states of India offer a great potential for freshwater prawn culture. Its farming has drastically increased within the last five years. The production in India is recorded to the tune of 42,820 tonnes. It is ranked second in the world[4]. Scampi is exported to European Union, Japan, USA and other countries. However, concomitant with the rapid expansion and intensification of scampi farming, the disease problems have been causing serious concern.

In any successful farming system, there should always be a proper balance between host, pathogen and environment. In intensive system or high input system, there is every chance of disease occurrence if there is no proper harmony among the three factors. India has already experienced the plummeting of most profitable shrimp aquaculture industry due to white spot disease. In recent years, due to adoption of unscientific and highly intensive farming practices, the freshwater prawn farming in the country has suffered a major setback due to occurrence of a viral disease, caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) [5-8]. It has occurred due to import of seed of *M. rosenbergii* from disease-affected countries. The virus has affected the hatcheries and nursery tanks in southern parts of the country causing huge economic losses[9]. This example demonstrates the possible dramatic and unpredicted consequences of transferring infected seeds. Although there is no report on the transfer of this pathogen to wild stock, it is most likely that the pathogen might have escaped to the wild. Therefore, the knowledge of the major diseases and defence mechanism of the freshwater prawn is the need of the hour to minimize or prevent the outbreak of such diseases and to ensure long term survival of scampi industry. In this contribution, an attempt has been made briefly to outline the major diseases and the defence mechanism in the giant freshwater prawn, *M. rosenbergii* de Man. which may be useful for the aquaculturists. The recent trends in scampi aquaculture are towards increased intensification and commercialization of production. Like other fish or animal species, with the increase in the aquaculture activities of scampi there is a likelihood of intensification of some major diseases, which are becoming the bottleneck for sustainability of this industry.

Major diseases of the giant freshwater prawn, *M. rosenbergii*

The giant freshwater prawn *M rosenbergii* de Man. is generally considered as a less susceptible species to disease problems as compared to penaeid shrimp. However, due to intensification of aquaculture practices and poor quarantine, various diseases, caused by pathogens and nutritional, physiological and environmental factors are often reported in hatcheries and farms. They seriously affect the commercial production. Some of the major diseases of the giant freshwater prawn are listed as follows:

VIRAL DISEASES

White Tail Disease (WTD)/White Muscle Disease (WMD)

The WTD was first observed in Guade Loupe island in 1995. Later on it was reported from Martinique island (French West Indies), Taiwan, Peoples Republic of China and India[5-13]. The disease is spreading rapidly causing worldwide huge economic loss to scampi industry. The pathogen was identified to be a virus, named as *M. rosenbergii* nodavirus (*Mr*NV). *Mr*NV is a small icosahedral non-enveloped virus, 26-27 nm in diameter, present in the cytoplasm of connective tissue cells. It has since been reported that *Mr*NV is associated with a small satellite virus, called extra small virus (XSV) [11, 12, 14, 15]. The disease affects hatchery-reared PL as well as nursery-reared early juveniles. The affected individuals show signs of whitish tail or milky-white muscle, leading to 100% mortality. The virus is transmitted vertically from brooders to larvae and PL[6]. Sudhakaran *et al.*[16] reported that marine shrimp act as reservoir host of *Mr*NV and XSV.

The clinical signs primarily indicate the disease but confirmatory diagnosis is done by transmission electron microscopy (TEM)[14]. Other diagnostic methods like double sandwich enzyme-linked immunosorbent assay (DS-ELISA) [14], triple antibody enzyme-linked immunosorbent assay (TAS-ELISA) [17], dot-blot hybridization, *in situ* hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR) [15, 18, 19], multiplex RT-PCR (MRT-PCR) [20, 21], nested PCR[22] and loop mediated isothermal amplification (LAMP) [23] can also be used for detection of *Mr*NV and XSV in scampi. Among this nested RT-PCR has been proved to be highly sensitive diagnostic technique, which has the detection

limits of 0.01 fg of total viral RNA corresponding to four viral particles[22].

Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)

Infectious hypodermal and haematopoietic necrosis virus is the smallest (approximately 22 nm diameter) of the known penaeid shrimp viruses[24, 25]. It is a non-enveloped, icosahedral virus[26]. The virus was first detected in the juveniles of *Litopenaeus vannamei* Boone. It causes acute epizootics with up to 90% mortality[27,28]. IHHNV also causes a chronic disease called runt deformity syndrome in *L. vannamei* Boone and *Penaeus monodon* Fabr.[29, 30, 31]. However, it was thought that the virus does not cause lethal infections in *M. rosenbergii* de Man. but recent reports of IHHNV infections in *M. rosenbergii* de Man. have shocked the scampi industry in Taiwan[32].

This emerging disease particularly encountered in postlarvae and juveniles of *M rosenbergii* de Man. causes 80%-100% mortality. Affected prawns show growth retardation and deformities of the fourth to sixth abdominal segments and telson, muscular atrophy, associated with reddish discolouration of the cuticle. Histologically, the striated muscles of the affected abdominal segments show Zenker's necrosis. Muscular lysis is also seen in the affected fibres. Nevertheless, no inclusion bodies are detected in any organ. The confirmatory diagnosis of this disease is performed by PCR assays and *in situ* hybridization[32].

White Spot Syndrome Virus (WSSV)

White spot syndrome virus (WSSV)/white spot syndrome baculovirus (WSBV) is the causative agent of a disease that decimated cultured penaeid shrimp populations and inflected severe economic damage throughout the world including India[25, 33-37]. Though *M. rosenbergii* de Man. was thought to be unaffected by this virus, but signs closely resembling WSSV have been observed on its exoskeleton[38]. Peng *et al.*[38] established WSBV as the causative agent of WSS in the giant freshwater prawn by using polymerase chain reaction (PCR) with WSBV-specific primers. Larvae, PL, juveniles and adults were found to be PCR- positive. The amplified PCR product was found to be similar to that of natural infected penaeid WSSV, hence great care should be taken to reduce or prevent the epizootics of WSSV in freshwater scampi culture.

In experimental studies by Rajendran *et al.*[37], a WSS virus obtained from *P. monodon* Fabr. from the south-east coast of India was used to infect *M. rosenbergii* but showed 100% survival and no clinical symptoms up to 70 days. In a similar study, Sahul Hameed *et al.*[39] used a viral inoculum obtained from *P. monodon* Fabr. with WSSV to test the relative susceptibility of three freshwater prawn species. All species tested had significant mortality except *M rosenbergii* de Man. even when the latter was given multiple injections of increasing dosage. In contrast, Kiran *et al.*[40] reported that the different life-stages of *M rosenbergii* de Man. are susceptible to WSSV.

Macrobrachium Muscle Virus (MMV)

Tung *et al.*[41] reported *Macrobrachium* muscle virus infection in *M. rosenbergii*, cultured in Taiwan. The virus is temporarily named MMV until its taxonomic position is ascertained by analyzing the genomic structure. This virus mostly affects postlarvae (PL) with epizootics similar to idiopathic muscle necrosis (IMN) syndrome with exceptional addition of dense cytoplasmic inclusion bodies in the affected muscles. Electron microscopy shows icosahedral virus particles of 22.9±3.6 nm size. MMV infects giant freshwater prawn in July and December within 10 days of moving PL to outdoor ponds. Moribund prawns grossly exhibited non-transparent muscle in abdominal segments 2-6. The affected prawns commonly displayed progressively weakened swimming ability and an inclination to stay on vegetation or the pond bank. A mortality of 50%-75% is seen within 2 weeks after transporting the postlarvae to grow-out ponds[41].

Macrobrachium Hepatopancreatic Parvo-like Virus (MHPV)

Macrobrachium hepatopancreatic parvo-like viruses have not received much attention among entrepreneurs and researchers because they are not causing any serious epizootics in *M. rosenbergii* culture. The definitive diagnosis of MHPV requires the histological demonstration of a characteristic inclusion body within the hypertrophied nucleus of the affected cells of the hepatopancreatic epithelia[42].

Macrobrachium HPV is similar to the hepatopancreatic parvo-like virus described from penaeid shrimp in terms of the target cells attacked, inclusion body characteristic and nucleic acid type. However,

it differs from shrimp HPV by an *in situ* hybridization test for genomic HPV that shows no positive reaction with MHPV preparations[43].

BACTERIAL DISEASES

Several diseases of bacterial origin are frequently reported in hatcheries and grow-out ponds. However, the type and levels of bacterial populations associated with farmed *M. rosenbergii* de Man are useful indicators of quality and safety of prawns[44]. It is reported that larval stages are most susceptible to a bacterial disease as a result of high stocking density, long rearing period and organic load accumulation at the pond bottom. Some of the bacterial diseases frequently encountered in scampi farming are as under.

Vibriosis

Vibriosis has been identified as a major challenge to scampi industry worldwide[43, 45-48]. Jayaprakash *et al.*[48] reported 9 species of Vibrio associated with *M. rosenbergii* larvae. The major *Vibrio* sp. infecting scampi includes *V. cholerae, V. nereis, V. alginolyticus, V. vulnificus, V. mediterranei, V. parahaemolyticus, V. splendidus, V. fluvialis, V. mimicus, V. harveyi* etc. Among these, *V. cholerae, V. alginolyticus* and *V vulnificus* are predominant[48, 49]. In general, *Vibrio* sp. are prevalent on eggs, larvae and post larvae of freshwater prawns[50] and their increasing number during culture period is a serious concern[51, 52].

It has been reported that luminescent bacterium *V. harveyi* causes massive losses of scampi larvae in hatcheries[53, 54]. The unique clinical sign of this disease is the luminescence of affected larvae that can be observed at night. Infected larvae also show fouling, opacity, slow swimming, aggregation and mortality. Mortality may reach 100% and may cause complete failure of the crop, resulting in severe economic loss to farmers.

The use of antibiotics to control vibriosis in shrimp/prawn hatcheries has been documented[55]. However, prophylactic use of antibiotics can lead to the emergence of antibiotic resistance in bacteria[56]. As an alternative management measure, the introduction of selected bacterial cultures/products as probiotics with antagonistic properties has been proposed and applied[57]. Misra *et al.*[58] reported that bath immunostimulation with ß- glucan provides better protection against *Vibrio* infection in *M. rosenbergii* de Man. Jayaprakash *et al.*[47] reported that marine bacterium, *Micrococcus* MCCB 104 show antagonistic properties against most of the *Vibrio* sp. associated with *M. rosenbergii* de Man.larval rearing system. Anas *et al.*[52] reported that chitosan can be used as a prophylactic biopolymer for protecting prawn larvae from vibriosis. Chitosan in the larval rearing system may function as a *Vibrio* growth depressant. As the risk of infection is directly related to pathogen density, depressed cell counts may help to prevent larval vibriosis. Further, chitosan is recognized as an immunostimulant in fish[59]; hence, it may also be worthwhile to test it as immunostimulant in freshwater prawn rearing system.

Shell Disease

Shell disease is the most common bacterial disease affecting crustaceans[60]. The disease has been variously referred to as brown spot disease, black spot disease and rust disease. Chitinolytic bacteria such as *Vibrio, Aeromonas, Pseudomonas, Bacillus* and *Benekea*, which produces extracellular enzymes, often have been associated with this disease[53, 61, 62]. The disease affects all life stages of *M. rosenbergii* de Man. The presence of one to several focal melanised lesions on the body surface is the most distinct gross sign[63]. Blackening is usually associated with melanin production that is bacteriostatic in action. The disease is usually seen focally on the gills, carapace, appendages, uropods, telson or body cuticle. The clinical signs are self-limiting and shed with the exuvia in healthy prawns. In severe cases, infection may spread to the epithelium, muscle and viscera resulting in septicemia and mortality[64].

Often, development of black spot is associated with poor husbandry, overcrowded conditions and poor sanitation[65]. Maintenance of optimum water quality, adequate feeding, less stocking density and hygiene are some important control measures. Antibiotic treatment has also been reported for control of black spot in *Macrobrachium* species[66]. Chemical treatment is not recommended for control of black spot disease in *M. rosenbergii* de Man[67]. Delves-Broughton and Poupard[68] however, viewed that shell disease does not lend itself to prophylactic and chemotherapeutics control. The best way to control shell disease in prawns thus seems to be thorough environmental management, which reduces stress factors and its regulation[69, 70].

Bacterial Necrosis

Bacterial necrosis, first described by Aquacop[69], is similar to black spot in terms of gross signs but

is restricted to larval phase only. Larval stages IV and V are generally more susceptible showing the necrosis and melanisation of whole body appendages. Other clinical signs of this disease are empty gut, bluish colour of affected larvae and brown spots on antennae and newly formed appendages. Such larvae gradually fall to the bottom of the tank. Mixed bacterial infections have been observed in such cases. Filamentous *Leucothrix* sp. and nonfilamentous bacilli and cocci usually infest on the setae, gills and appendages of the affected freshwater prawns[71].

Bacterial necrosis in *Macrobrachium* larvae may represent a secondary bacterial invasion of the cuticle following some as-yet-undetermined primary insults, which cause disruption of the epicuticle allowing bacterial entry[67]. A diagnosis of bacterial necrosis in larvae of freshwater prawn is made by microscopic demonstration of the characteristic focal to multifocal melanized cuticular lesions of larval appendages[72].

Development of bacterial necrosis is rapid and if untreated, can be responsible for mass mortality of larvae within a short span of time. Onset of necrosis has been linked to a decrease in larval resistance[73]. Antibiotic treatment has been reported to be effective for control of bacterial necrosis in *Macrobrachium* larvae[71].

Gram-positive Bacterial Infection

Very few reports are available on infection of Gram-positive bacteria in cultured freshwater prawn *M. rosenbergii* de Man. Brock *et al.*[74] diagnosed Gram-positive bacteria *Mycobacterium* in a female broodstock kept in captivity for more than 7 years. Cheng and Chen[75] isolated *Enterococcus*-like bacterium from the musculature of *M. rosenbergii* de Man in Taiwan, which has been identified as *Lactococcus garvieae* by polymerase chain reaction assay and 16s rDNA sequencing[76]. Such infected prawns show necrosis of tissue in the hepatopancreas and musculature. Experimentally it has been proved that high pH and temperature exacerbate infection while low salinity appeared to have a favourable effect upon survival[77].

RICKETTSIAL DISEASE

Cohen and Issar[78] reported rickettsial infection of the hepatopancreas of *M. rosenbergii* de Man larvae at stage IV to V resulting in 40% to 95% mortality. Microscopically, the disease was characterized by atrophy of the hepatopancreas[79]. The infection was successfully transmitted to healthy larvae by exposure to a homogenate of infected larvae[78]. Application of calcium oxide (CaO) at 10 to 15 ppm reduces losses, as did treatment with 10 ppm of either terramycin or furazolidone[79, 80].

Considering the frequency of its infection in crustaceans, it is apparent that rickettsial diseases are potentially more important than was once understood[63].

FUNGAL DISEASES

Fungal infection of the Phycomycete *Lagenidium* in *M. rosenbergii* de Man.hatcheries causes decimation of larval populations in less than 24 h[81]. The infection can be readily diagnosed by the observation of an extensive mycelial network visible through the exoskeleton of infected larvae. It is reported that Treflan at 10-100 ppb is effective in controlling of *Lagenidium* infestation in penaeid hatcheries[82].

Recent work with a large sampling of isolates in Taiwan has helped to remove confusion about the identity of prawn-infecting yeasts[83, 84]. Yeasts reported to be pathogenic for *M. rosenbergii* de Man.include *Candida sake* I and II, *Pichia anomala, Endomyces fibuliger* and *Candida famata*[85]. Su *et al.*[84] reported that yeast *Metschnikowia bicuspidata* is the major cause of white muscle disease, but the molecular mechanism of its pathogenesis is not known. The imperfect fungus, *Fusarium soleni, Saprolegnia, Sirolopidium* and other fungal genera may also be associated with prawn mortality[82, 86]. The yellowish, bluish or greyish colour of the musculature and a reduced resistance to the effects of stressful conditions, are indicative of such diseased animals[87]. The occurrence of the disease has been associated with the accumulation of organic matter in the pond bottom and eutrophication in grow-out ponds. Therefore, possible preventive measures are careful pond preparation, proper feeding, water exchange and aeration etc, which provide congenial environment for freshwater prawn culture.

PARASITIC DISEASES

Microbial Fouling

Microbial epibionts are commonly observed disease agents in *M. rosenbergii*. Epibiont fouling organisms include filamentous bacteria, algae or protozoa common

to the aquatic environment. The presence of epibionts, nonetheless, can be deleterious to freshwater prawn. Fouling by various microorganisms can provoke natatory and feeding impairment during larval and postlarval phases. Heavy infestation of filamentous algae, protozoans and miscellaneous microbes over the body surface of reared animals in clear ponds, diminish the market value of the prawns. Heavy fouling by the cholorophyta *Oedogonium* and the cyanophyta *Lyngbya* can cause lethargic behaviour in *M rosenbergii* de Man[88].

The most common protozoans found in freshwater culture are the peritrich ciliates *Zoothamnium, Epistylis, Vorticella, Opercularia, Vaginicola, Cothurnia* and *Lagenophrys* and the suctorians *Acineta, Podophrya, Tokophrya* and *Ephelota*[89, 90]. Larvae of scampi are more susceptible to protozoan infections than adults. These organisms are non-invasive and their presence on the cuticle surface does not trigger the activation of the proPO system, unless a localised mechanical trauma is present. Generally, *Zoothamnium* sp., *Epistylis* sp., *Vorticella* sp. and *Acineta* sp. cause slight opaqueness in body colour of freshwater prawn. Shedding of the exoskeleton during ecdysis temporarily frees the prawn from these fouling microorganisms. Large animals and blue claw males, which moult less often, are frequently seen with excessive burdens of epibionts. Improvement of water quality is necessary when the outbreaks occur. Treatment with 20-30 ppm formalin is effective and safe in controlling *Zoothamnium* infection of the larvae[90]. Repeated treatment of 2 ppm acetic acid as a 1 min dip is recommended for *Epistylis* sp.[91].

Metazoan Parasites

The infection of metazoan parasites in cultured freshwater prawn is rare. Most reports concern over the occurrence of parasitic isopods and trematode worms in natural populations of other *Macrobrachium* species. Isopods that infest freshwater prawns belong to the family Bopyridae[92]. The asymmetrically shaped female bopyrid is found attached to the branchiostegite wall within the gill chamber and feeds on the host haemolymph by perforating the integument with the help of its mandibles. The symmetric free living dwarf males are found associated with the parasitic females. The presence of the parasite in the gill chamber usually induces a visible swelling and discolouration on the branchiostegite[93]. Johnson[94] reported that *Macrobrachium* serves as second intermediate host for the metacercaria of *Carnaeophallus choanophallus*. Nash[95] reported the only known trematode infection in *M rosenbergii* de Man. musculature and described the formation of a haemocytic capsule around the parasite as the result of defence response. Control methods are limited to physical removal of parasites from prawns and prevention of extension of the range of these parasites into new geographic area.

OTHER DISEASES OF UNKNOWN AETIOLOGY

Appendage Deformity Syndrome (ADS)

Recently, a new disease with unusual clinical signs has been reported in the Nellore district, motherland of scampi culture in India. This disease is named appendage deformity syndrome (ADS) because of the physical appearance of affected prawns[7, 96]. ADS has affected more than 80% of the area under culture in Nellore district.

ADS is a chronic disease associated with late mortality particularly at 4 to 6 months of culture when prawns have reached maturity. The clinical signs of ADS are more prevalent in females than males. The most striking features of the disease are appendage deformity, broken, bent or deformed rostrum, cut antennae, beaded or corrugated appearance of antennules which are more prone to breakage, corrugated appearance of the carapace, poor growth and varied mortality[7, 97]. Kumar *et al.*[96] reported that carotenoid supplementation (1g/kg) in the diet significantly reduces the ADS in prawns, both in laboratory and field. The same study also ruled out possibility of involvement of any bacterial or viral agents responsible for causing ADS. Sahoo *et al.*[7] reported that alterations in the physico-chemical parameters of water might be playing roles as predisposing factors or causal factors for ADS. However, further study is required to confirm the involvement of any pathogen or carotenoid deficiency or water quality as the possible cause of ADS.

Branchiostegal Blister Disease (Balloon Disease)

Pillai *et al.*[98] reported a branchiostegal blister disease or balloon disease of farmed *M. rosenbergii* de Man. restricted to Nellore region of India. The disease is clinically characterised by the swelling of the gill flap or branchiostegite region and is hence named as 'Branchiostegal blister disease' (BBD) ' or ' balloon disease' by the farmers[99]. It usually appears in nurseries within

30 days of stocking or in grow-out ponds after transfer from nurseries. The mortality ranges from 70% to 80%. The cause of the disease is unknown but it is thought that suboptimal water quality conditions may be responsible for it.

Idiopathic Muscle Necrosis

Idiopathic muscle necrosis (IMN) is a pathological condition reported from cultured *Macrobrachium* larvae, PL, juveniles and subadult[64, 100, 101]. This disease is also known as white muscle disease, spontaneous muscle necrosis, muscle opacity or milky prawn disease. It causes massive larval mortalities in hatcheries[53,64]. The disease shows focal and multifocal diffused opacity of striated muscles. At the chronic stage of the disease, the necrotic affected area may increase in size and acquire a reddish colour, similar to cooked shrimp, due to decomposition of the muscle tissue. Prawns suffering from chronic IMN do not survive. The mortality rates in culture populations are reported to vary from insignificant to 100%[67].

Diagnosis of IMN in *Macrobrachium* larvae can be made by gross demonstration of opacity of the abdominal musculature[102]. Histopathology is suggested for confirmation of the characteristic focal degenerative and/or regenerative change in the musculature. A specific approach for the control of IMN in *Macrobrachium* zoea is presently not documented. The development of the disease appears to be associated with poor management. IMN has been observed in populations submitted to high stocking rates and stressful environmental conditions such as low dissolved oxygen level, salinity and temperature fluctuations[1, 53]. The obvious preventive measures include the application of good husbandry to avoid IMN inducing stressors.

Larval Mid Cycle Disease

A syndrome of *Macrobrachium* larvae with the onset in the middle of the larval cycle is mid-cycle larval disease (MCD). It has been reported from Hawaii, Malaysia and Brazil[42, 103]. Although the implication of many pathogens, toxic compounds or pesticides have been suggested, a precise cause remains undetermined[103]. Mid-cycle disease is probably a disease of multiple aetiology.

The onset of MCD usually occurs by 15 to 22 days when zoea larvae are at stage VI-VII[103]. Clinical signs include lethargy, a spirally swimming pattern, reduced growth rate, less consumption of *Artemia*, bluish-grey colour and atrophy of the hepatopancreatic epithelium. Larvae lose their appetite. The healthy ones eat moribund individuals showing cannibalism. Destroying the stock, strict attention to hygiene and sanitary conditions are some of the control measures. Brock[67] reported that reducing the stocking density and improving pond husbandry and sanitation could control the MCD disease.

Exuvia Entrapment Disease

The exuvia entrapment disease (EED) is also known as moult death syndrome (MDS) or metamorphosis moult mortality syndrome. It is characterised by the entrapment of the larvae in the old exoskeleton during ecdysis. This primarily occurs late in the period of larval rearing cycle and most commonly at the metamorphic moult from stage XI to post larvae[67]. Affected larvae are unable to free their appendages, eyes or rostrum from the exuviae during and after moulting and become entrapped. Mortality occurs during unsuccessful attempts by larvae to shed the exuvia. It may reach 80% by the time of metamorphosis[67]. Kanaujia *et al.*[104] reported an exuvia entrapment like disease in Indian river prawn *M. malcolmsonii* Milne Edwards, causing mass mortality in hatchery tanks.

The presumptive diagnosis of EED in *Macrobrachium* larvae is made by high survival up to stage X-XI, many of moribund or dying larvae/PL in late premoult, entrapped in their exuviae, moulted larvae showing severe, lethal appendage deformity which may have apparently stalled in a supernumerary stage XI[67].

The precise cause of EED is unknown but it is thought that poor water quality, and inadequate nutrition may be responsible for this dreaded disease. Preventive strategies should include use of a new water supply of more suitable quality and dietary enrichments. The moulting process creates severe physiological stress and during this period the disease manifests itself.

Black Gill Disease

Johnson[89] reported that precipitating chemicals and nitrogenous waste matter present in the water are responsible for melanization of the gills. High amount of ammonia and nitrite in the ponds result in growth suppression and mortality. *Macrobrachium* are more

susceptible to high nitrite and nitrate concentrations than penaeid shrimps[105]. Sublethal effects of nitrite could be fatal in chronic exposure and may occur at less than 2 ppm[106]. Nitrogenous compound levels in rearing water medium should be routinely monitored. Preventive measures should therefore include feeding adjustment or increased water replacement in rearing tanks or grow-out ponds.

Red Discolouration

Johnson[92] reported reddish abdominal discolouration affecting adult freshwater prawn in a grow-out pond. The abnormal pigmentation had resulted from the movement of the pigment from chromatophores that had lost their usual integrity. The precise cause of red discolouration is not known but it is thought that excess light, diet and stress may be responsible for this abnormality.

Defence mechanism of the giant freshwater prawn, *M. rosenbergii*

The defence mechanisms of *M. rosenbergii* de Man. and other crustaceans differ from those of the vertebrates. It does not show the production of immunoglobulins. Defence system of giant freshwater prawn may be classified as fixed defences, mobile defences and Pro-phenol oxidase system (PPO) defence. The hard exoskeleton or cuticle of freshwater prawn acts as structural and chemical barrier to several pathogens. It acts as a first line of defence and has the capacity of rapid wound healing which prevents loss of haemolymph. It contains immune substance like PPO[107]. Humoral (antisomes) and cellular (haemocytes) factors, which involve the direct participation of circulatory haemocytes and dissolved factors in the plasma, regulate the mobile defence system. Although the defence response in crustaceans lacks the antigen-antibody specificity found in vertebrates, it is nonetheless efficient in recognizing the antigen and taking prompt action to immobilize and eliminate invading microorganisms[63].

M. rosenbergii de Man.has an open circulatory system with no equivalents to vertebrate red blood cells, but cells analogous to the white blood cells appear to exist. These cells, called haemocytes, can be grouped into three subpopulations in crustaceans: granular, semigranular and hyaline cells[108, 109]. The circulating haemocytes of freshwater prawn play an extremely important role not only by direct sequestration and killing of infectious agents but also by synthesis and exocytosis of a battery of bioactive molecules[110, 111]. Essentially, the haemocytes execute inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites and the release of microbicidal protein[112, 113]. In crabs, the hyaline cells mainly execute phagocytosis and production of reactive oxygen while in other species, semigranular cells may be phagocytic. *Marsupenaeus japonicus* Bate. is unusual as in this species the granular cells are phagocytic instead of hyaline cells[114, 115]. There exists difference of opinion on the cell types and their phagocytic function in *M. rosenbergii* de Man. For example, Sung *et al.*[116] found all the three cell types (hyaline, semigranular and granular cells), in haemolymph of *M. rosenbergii* de Man. which display atleast some phagocytic activity. Based upon the recent classification of *M. rosenbergii* de Man.haemocytes[117, 118], large ovoid haemocytes and undifferentiated round haemocytes might be carrying out the functions of the proPO system, similar to semigranular and granular haemocytes of other crustaceans[119].

Phagocytosis can be initiated by receptor-mediated endocytosis or through non-specific hydrophobic interactions of cell membrane with target particles[120, 121]. It is a common reaction of cellular defence and is generally recognized as a central and important way to eliminate microorganisms or foreign particles. Phagocytosis includes attachment to the foreign body followed by its ingestion and destruction[122, 123]. In phagocytosis, a form of endocytosis, large particles move into endocytic vesicles called phagosomes. Prawn haemocytes contain cytoplasmic granules and lysozyme[124]. A wide variety of degradative enzymes and antimicrobial peptides are released into the phagolysome following intake of foreign organisms. These enzymes include proteases, nucleases, phosphatases, esterases and lipases[125].

Several kinds of reactive oxygen intermediates (ROIs) are produced during phagocytosis. Once the invading microorganism infects the host, it activates the host's NADPH-oxidase, which in turn reduces oxygen molecules and subsequently produces several ROIs such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) and singlet oxygen ($^1O_2$) [109]. These compounds can be toxic to the pathogens[126]. This phenomenon, known as respiratory burst activity plays a vital role in microbicidal

activity[127]. The generation of superoxide anion has been reported in the haemocytes of the shore crab, *Carcinus maenus*[128], the tiger shrimp, *Penaeus monodon*[127] and the blue shrimp, *P stylirostris*[70], white shrimp *L. vannamei*[129-131] *Marsupenaeus japonicus*[114], *M. malcolmsonii*[132, 133] and *M rosenbergii*[118]. It is also reported that rapid increase of superoxide anions and other ROIs may pose potential cytotoxic problems to the host[130, 134]. Antioxidant defence mechanisms including NADPH oxidase, superoxide dismutase (SOD), peroxidase and catalase perform the effective and rapid removal of ROIs[135,136].

Superoxide dismutase is an important antioxidant enzyme that scavenges superoxide anion and converts it to molecular oxygen and hydrogen peroxide[135]. They are classified into three distinct groups depending on the metal contents: iron SOD (Fe-SOD), manganese SOD (Mn-SOD) and copper/Zinc SOD (Cu, Zn-SOD). Fe-SOD has been found in prokaryotes and in plants [137]. In decapod crustaceans, cystolic Mn-SOD (cytMn-SOD) has been identified in tiger shrimp, *P. monodon* Fabr. (GenBank accession no. AAW50395), kuruma shrimp, *M japonicus* (GenBank accession no. BAB85211), brown shrimp, *Farfantepenaeus aztecus* Ives., grass shrimp, *Palaemontes pugio* Holthius, blue crab, *Callinectes sapidus* Rathbun.[138] and *M rosenbergii* de Man[136]. Mn-SOD has been cloned in lobster, *Palinurus vulgaris*[139], white shrimp, *L. vannamei* Boone.[140], tiger shrimp *P monodon* Fabr. (Genebank accession no. B1784454), kuruma shrimp, *M. japonicus* Bate. (Genebank accession no. BAB85211), grass shrimp, *Palaemontes pugio* Holthuis. (Genebank accession no. AY211084), blue crab, *Callinectes sapidus* Rathbun.[138,141] and *M. rosenbergii* de Man.[142] However, in crustaceans, little is known on the cloning and characterization of Cu, Zn-SOD. Recently, Cu, Zn-SOD has been cloned from the hepatopancreas of *M. rosenbergii* de Man.[143]

In addition to phagocytosis, haemocytes also enhance coagulation and encapsulation. They accelerate the production of melanin via the prophenoloxidase system[144, 145]. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which is similar to capsule formation, occurs when the number of invading bacteria is high[109]. The prophenoloxidase (proPO) activating system is a cascade of enzymes and other proteins, which is contained in the granular and semigranular cells, and can specifically be activated by several microbial polysaccharides, including β-1,3-glucan from fungal cell walls[146-148] and peptidoglycans[149] or lipopolysaccharides[150] from bacterial cell walls. Additional factors found to activate the proPO system include calcium, sodium dodecyl sulfate (SDS), trypsin, and high temperature[149, 151-154]. According to Ashida and Soderhall[151], these elicitors activate the proPO system through different mechanisms.

The major enzyme produced during proPO system activation is phenoloxidase (PO) that is necessary for the melanization process observed in response to infection and occurs in cuticular wounds or nodules and capsule formation around invading parasites[144]. A complex enzymatic cascade that involves activation of proPO to PO induces melanization[123, 155]. PO is a copper-containing oxidase that catalyses the oxidation of phenolic substances such as L-3, 4- dihydroxyphenylalanine (L-DOPA) to quinones which are then further polymerized non-enzymatically to the black pigment, melanin[156]

Without doubt, the proPO system is a dominant part of the crustacean defence system exerting effects on cell behaviour, liberation and/or activation of functionally important molecules and neutralization of infective agents. Its multiplicity of effects, direct or indirect, not surprisingly, has made it one of the central parts of the crustacean immune system to be targeted for up-regulation by externally administered stimulants[113]. The activity of phenoloxidase has been reported from many crustaceans including the brown shrimp, *Penaeus californiensis* Holmes.[157], Sao Paulo shrimp, *Farfantepenaeus paulensis* Perez-Farfante.[158], white shrimp, *Litopenaeus vannamei* Boone.[129, 131], tiger shrimp *P. monodon* Fabr.[159], Indian river prawn, *M malcolmsonii* Milne Edwards.[132, 133] and giant freshwater prawn, *M. rosenbergii* de Man.[118, 145].

Several compounds or associated factors of the proPO system have been found in crustaceans. They include the recognition proteins[160, 161], cell adhesive proteins[162-164], protease inhibitor[165] and proPO activating proteinase[166, 167]. The biological activity of peroxinectin, a cell adhesive protein, is generated concomitant with the activation of the proPO system[168]. During cell adhesion, peroxinectin is synthesized and stored in semi-granular and granular haemocytes in an inactive form. It is released in response to a stimulus and activated outside the cells to mediate haemocyte attachment and spread[168-170]. Peroxinectin has multiple functions of degranulation[119], encapsular enhancement[171], opsonin[172]

and is related to the activity of peroxidase[162]. When a foreign particle enters the haemolymph of a host, haemocytes recognize the foreign intruder as non-self and change from non-adhesive cells to adhesive cells to strongly adhere to the foreign target. The semigranular and granular cells attach and spread across the surface of the foreign intruder and subsequently form a multilayered sheath of cells during encapsulation. Therefore, peroxinectin is essential in crustacean cellular defence reaction for enhancement of encapsulation and phagocytosis[119].

Peroxinectin has been isolated and purified from crayfish, *Pacifastacus leniusculus* Dana and tiger shrimp, *P monodon* Fabr. and show cell adhesion and peroxidase activities in the presence of lipopolysaccharide (LPS) or β-1,3-glucans[163, 168], suggesting that this protein plays an important role in defence against pathogens[170]. Peroxinectin cDNA has been cloned and sequenced from the haemocytes of *P. leninusculus* Dana[162], *P. monodon* Fabr.[163], *L. vannamei* Boone[170] and *M. rosenbergii* de Man[173].

A number of haemolymph factors are associated with defence reaction of *M. rosenbergii* de Man. These factors include naturally occurring or inducible bioactive molecules, which agglutinate and precipitate the non-self particles as well as those that have bactericidal, lytic or bacteriostatic properties. Agglutinins, which cause aggregation or agglutination of foreign particles, have been reported in freshwater prawn[132, 133]. Agglutinins would help sequestration of invasive organisms from the haemolymph and therefore contribute to disease resistance[174]. Lectins, which are generally recognized by their ability to cause haemagglutination are also reported from many crustaceans species including *M. rosenbergii* de Man[175-181] and *M. malcolmsonii* Milne Edwards[132, 133]. Lectins are multivalent carbohydrate binding proteins that actively participate in cellular and humoral immune response[182, 183] and are able to agglutinate erythrocytes, bacteria and other cells through interaction with appropriate complementary ligands[184-186]. In *M. rosenbergii* de Man. a homodimeric protein of 9.6 kDa per subunit (MrL) has been purified in a single step by affinity chromatography on a column with stroma from rat erythrocytes[187]. It is specific for N-acetylated sugar residues, such as N-acetyl-D-neuramic acid (Sialic acid, Neu5Ac), N-acetyl-D-galactosamine (GALNAc) and N-acetyl-D-glucosamine (GLcNA)[187] and shows high affinity for sialylated glycoproteins and glycans[188]. Agundis *et al.*[178] quantified lectin in *M. rosenbergii* de Man. haemolymph by ELISA. Soria *et al.*[181] reported that sugars and amino acids present in the haemolymph of prawn also regulate lectin activity and the oxidative burst. It has also been reported that *M rosenbergii* de Man. lectin (MrL) agglutinates erythrocytes from rat and rabbit, and possess the capacity to agglutinate bacterial species with well known polysaccharide determinants such as *Pasteurella* hemolytic serotype A (Capsular serotype 12), *Bacillus cereus* and *Aeromonas* sp., as well as in a minor proportion, *Escherichia coli* and *Salmonella arizona*[188]. MrL has also been identified as a marker for neuro-degenerative diseases, such as Alzheimer disease[189].

Clotting factors play a vital role in defence system of crustaceans. Coagulation in crustaceans results from the direct conversion of a soluble fibrinogen (coagulogen) into crosslinked fibrin through the action of a coagulin released by the haemolymph[110]. The activation of clotting cascade may be triggered by microbial cells such as LPS and β-1,3-glucans, resulting in the formation of a clot that entraps invading microorganisms. This process is also linked to the triggering of proPO activation[123].

Strategies for combating diseases in prawn aquaculture

For preventing or controlling infectious diseases in prawn farming, the following possible strategies are to be followed.

- India should implement quarantine rules/protocols stringently and an established international aquatic animal health code on the introduction and transfer of organisms must be followed.
- Asian Regional Technical Guidelines (FAO[190]) on health management for the responsible movement of live aquatic animals should be implemented.
- The national strategy on listing pathogens, developing diagnostics, health certification, seed certification, quarantine, hatchery accreditation, institutional-governmental-farmer cooperation may be strengthened.
- Level I diagnostic facilities at the field level (gross examinations), level II diagnostics at state levels (use of parasitological, bacteriological, mycological and histopathological tools) and level III diagnostics at research institute/university/central level (i.e.

laboratory observations using virology, immunology, molecular biology and electron microscopy) should be strengthened.

Biosecurity, disease surveillance, reporting and disease zoning may be planned.

Research should be strengthened with respect to development of easier, sensitive and rapid diagnostics; epidemiological mapping of prawn diseases with special emphasis on interventions, risk factors, forecasting and disease modeling; determining pathways for introduction, establishment and spread of pathogens and understanding detailed pathology of the diseases.

Nevertheless, emphasis should be given on manpower development with respect to education, training, extension and diagnostic services and establishment of regional reference laboratories to cater the need of the prawn farmers.

Conclusions

The intensive culture practices of *M. rosenbergii* de Man.occasionally trigger the development of disease outbreaks, which are often explosive and sometimes lead to the loss of entire crop. In recent years, India and many countries around the world have experienced the devastating effect of the viral diseases, caused by nodavirus and extra small virus on production system, resulting in losses of several million dollars and identified as a major constraint to the rapid spread of this high-profit industry[191-193]. Hence, knowledge of major diseases and the defence mechanism of freshwater prawn are crucial for sustainable development of scampi industry. Majority of disease conditions observed in cultured freshwater prawns are caused by a combination of pathogenic, nutritional, physiological and environmental factors. Proper management of water quality and well balanced diet will immensely help in reducing such infections. Furthermore, immunostimulants like β-glucan, lactoferrin etc, which build up protection against broad spectrum of pathogens, become increasingly important to enhance the immune status of freshwater prawn[58, 118, 194] after realization of the side effects of antibiotics treatment and other chemotherapeutics. In cases, where disease outbreaks are cyclic and can be predicted, immunostimulants may be used in anticipation of events to elevate the defence mechanism and thus prevent further losses from disease. In addition, provision of quarantine facility at the farm site, also helps to prevent the transfer of disease agents with live aquatic animal movements[195]. Quarantine programmes that form part of the first line of defence against possible adverse effects resulting from the introduction or transfer of exotic fish and shellfish should be given importance.

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Impact of trawling on resource partitioning among certain demersal fishes of the Kerala coast

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Abstract

Bottom trawling is an important fishing method for capturing prawn and other bottom dwelling fishes which has also resulted in worldwide concern on its adverse impact on the marine fauna in general and bottom fauna in particular. There are differing opinions regarding the indirect impact of trawling on benthic fauna. According to some studies, trawling may also result in enhanced growth rate of fishes by increased availability of food The present study is undertaken to investigate the change in resource partitioning among three demersal fishes due to trawling viz. *Nemipterus japonicus* (Bloch), *Priacanthus hamrur* (Forsskal) and *Nibea maculata* (Bloch and Schneider). Quantitative analysis of stomach contents, based on several statistical indices including diet overlap, diet breadth and index of relative importance indicate the change in dietary patterns of the fishes Results of the study revealed interesting diet sharing patterns among the selected demersal fishes at trawled and non-trawled sites.

Key words : resource partitioning, dietary patterns, demersal fishes, trawling.

सारांश

खेचू जाल से मछली फसाना मछली पकडने का एक प्रमुख ढग है। इस तरीके से झीगा और दूसरी समुद्रतल में रहने वाली मछलियो को पकडा जाता है। पूरे विश्व में यह एक चिंता का विषय है क्योकि इस ढग से मछली पकडने पर लगभग पूरे, विशेषकर समुद्रतल मे रहने वाले, जीवो पर प्रतिकूल प्रभाव पडता है। इस सदर्भ मे वैज्ञानिको के भिन्न–भिन्न मत है। कुछ के अनुसार इस व्यवस्था से भोजन की उपलब्धता बढ जाती है, जिससे मछलियो मे बढी हुई वृद्धि दर देखी जा सकती है। प्रस्तुत अध्ययन मे इसका प्रभाव जिन तीन प्रजाति की मछलियो मे देखा गया वे है, *नेमिप्टरस जैपोनिकस (ब्लाच), प्रियाकैन्थस हैमरक (फोर्स्कल) तथा नीबिया मैक्युलाटा (ब्लाच तथा शेनेडर)*। इन मछलियो के अमाशय मे मौजूद साख्यकीय अनुसूची पर आधारित आहार अतिव्याप्त तथा आहार विस्तार के मात्रात्मक विश्लेषण से इनके भोजन पद्धति मे परिवर्तन इगित होते है। इस अध्ययन का परिणाम, चयनित अधोजलीय मछलियो मे दोनो स्थानों से, जहॉ महाजाल का प्रयोग हो रहा है या नही, रूचिकर, आहार सहभागी प्रतिरूप प्रकट करता है।

सांकेतिक शब्द : युक्ति विभाजन, आहार पद्धति, डिमर्सल मछलियॉ, जाल द्वारा मछली पकडना।

Introduction

Bottom trawling is an important fishing method for capturing prawn and other bottom living fishes. This method has resulted in worldwide concern regarding its adverse impact on the marine fauna in general and bottom fauna in particular. The impacts due to trawling have been noticed to be both physical and biological. The otter trawl, which alone is in operation in Kerala, has been reported to cause scraping, penetration and pressure of gear parts on the sediment, creating sediment suspension. These physical impacts result in habitat disturbance, mortality of bottom fauna, and exposure of bottom animals to predators and large-scale destruction of non-target species. There had been concerns regarding the decline in fisheries due to obstruction of spawning and large scale destruction of spawners of fin fishes and prawns during monsoon season. This has led to the implementation of trawling ban during monsoon throughout Kerala coast. Several studies in Europe have revealed that long term trawling may affect and shift community composition of macro benthos[1]. Most of these studies were indirect. Only a few direct observations have been reported.

However, there are differing opinions regarding the indirect impact of trawling on benthic fauna. Trawling[2] may also result in enhanced growth rate of fishes by increased availability of food. Benthic predators may be benefited by the shift in species and size composition of the bottom fauna.

The present study was undertaken to investigate the change in resource partitioning among three demersal fishes due to trawling.

Material and Methods

The fishes selected for the present study were *Priacanthus hamrur* (Forsskal), *Nemipterus japonicus* (Bloch) and *Nibea maculata* (Bloch and Schneider). These fishes live near the bottom upto 20-60m of depth and are present throughout the western Indian Ocean. They feed on a wide range of bottom living animals including worms, crustaceans, cephalopods and fishes. The diet changes little with size, as small fishes prefer small crustaceans (copepods and ostracods).

Two study sites were selected, one a well known trawling ground (Neendakara) and another an important fish-landing centre (Vizhinjam) which is basically a non-trawling site. Vizhinjam, is a small village situated about 16 km south of Trivandrum (Long 76° 59' 15" E, Lat. 8° 22' 30" N) is an important fish landing centre among the twenty seven fish landing villages in the fishery zone. The Vizhinjam coast is characterized by rocky inshore realms coupled with offshore areas of hard bottom with shallow ridge-like features which rise 2-5 m from the ground level, with an irregular profile. Of the four principal fishing methods used, fishing by trawls is not practised at Vizhinjam due to the rocky nature of the sea bottom. The methods practised in this coast are the use of seines, driftnets and hook and lines. Among the various gears employed at Vizhinjam, the most important one that contributes to the bulk in perch landings is the hook and line method in the mechanised sector. Most of the sciaenid catch obtained by konchu vala and boat seine is *Nibea maculata.*

The fishing village of Neendakara is situated 9 km north of Quilon (8° 53'- 9° 02' N and 76° 31' - 76° 41' E) on the banks of Ashtamudi channel which connects Ashtamudi lake with the sea. The sea off Neendakara is a potential trawling ground for prawns and other ground fishes. This area is reported generally to be sandy and muddy.

As seen from the fishing operations using otter trawl net and gill net, the CPUE for trawling is always higher than that for gill netting[4].

Monthly samples were collected from the trawl catches of Neendakara fishing harbour and from the fish landing centre at Vizhinjam for a period of one year from August 2001 to July 2002.

The stomach contents were analyzed by employing points (volumetric) method. Since volumetric method alone is inadequate to give a correct picture of the dietary importance[5], both occurrence and volume have been taken into consideration.

The percentage of each category of stomach to the total number of stomachs examined in a month was calculated and these values stand for feeding intensity. With a view to assess the feeding intensity, 'feeding index' was worked out according to the procedure of Kow[6].

Volume of each food item and its percentage in the total volume of all stomach contents were calculated monthwise by points method. The percentage occurrence of different food items was determined monthwise from the total number of occurrence of all items. To evaluate the importance of each food item, the 'index of preponderance' proposed by Natarajan and Jhingran[5] was followed. This method simultaneously takes into account both volumetric as well as occurrence methods in respect of individual food elements based on their relative importance. Bhatnagar and Karamchandani[7] adopted and modified the above method for grading. The formula for calculating the index of preponderance is V+O/2 where 'V is the percentage composition and 'O' is the percentage occurrence.

The gastrosomatic index (Ga.SI) was calculated using the formula,

$$\text{Ga.SI.} = \frac{\text{Weight of the gut}}{\text{Weight of the fish}} \times 100.$$

Coefficient of condition or condition factor was determined by the Fulton's formula.

The data from the dietary analysis were used to calculate diet breadths, using the niche width index (B) determined by Levins[8].

$$B = [\ \Sigma\ Pi^2\ ]^{-1}$$

where Pi is the proportion of the i-th item in the diet. This index was used to compare 'specialist' tendencies between species, with low values of the index indicating 'specialists' and high values, 'generalists'. The values of B increase from 1 as the diet becomes

broader. Diet overlap was estimated by comparing the proportion of diet for pair wise species (Schoener[9]).

$$\alpha = 1-0.5 \left(\sum_{i=1}^{n} |P_{xi} - P_{yi}| \right)$$

where n is the number of food categories, P_{xi} = proportion of food category i in the diet of species x, and Pyi = proportion of food category i in the diet of species y.

Dietary similarity of the same species of fish between Vizhinjam and Neendakara is computed using the following index :

$$S = \frac{\sum_{i=1}^{S} \min (P_{ix}^{V}, P_{ix}^{N})}{\sum_{i=1}^{S} \max (P_{ix}^{V}, P_{ix}^{N})}$$

where P_{ix}^{V} and P_{ix}^{N} are the proportion of the i-th item of species x at Vizhinjam and Neendakara respectively, and S is the total number of food categories. This formula is a modified form of Czekanowski's Quantitative index. Here the percentage composition of the i-th food category has been used, and in the index, the minimum value is normalized with respect to the maximum value. The index takes the value '0' when the diets contain no items in common between the two stations, and it takes the value of 1 when the diet contains identical items. So this index gives an indication of similarity in the dietary components of the same fish at two locations.

Asymmetrical diet overlaps were calculated using MacArthur and Levins[10] and Wallace[11] formulae:

Levins and Horn's formulae are:

$$\phi_{ij} = \frac{\Sigma_{i=1}^{S} p_{ik} p_{ij}}{\Sigma_{i=1}^{S} p_{ij}^{2}}$$

$$\phi_{ijk} = \frac{2.\Sigma_{i=1}^{S} p_{ik}\, p_{ij}}{\Sigma_{i=1}^{S} p_{ij}^{2} + \Sigma_{i=1}^{S} p_{ik}^{2}}$$

where P_{ik} is the percentage composition of the food item i consumed by the fish k and p_{ij}, is the % composition of the food item i consumed by the fish j at the same site. The pair (j, k) is taken as any one of the pairs of fishes. These measures reflect the differences in the magnitude of overlap between two fish populations due to unequal niche breadths. These indices are computed for a pair of fish at the same site. This asymmetrical measure can be viewed as a reflection of the unequal consumption of food items available at the same site by two competing fishes.

The quantitative importance of each component in the diet was determined by the index of relative importance (IRI)[3,12,13] which is defined as IRI=%F (% N + % V) where % F = frequency of occurrence of the food item, % N = numerical percentage of a food item in the stomachs, and % V = percentage by volume of the food item in the stomachs. In the present study, %F was taken as the % occurrence of a food item, and % N as the average composition of a food item.

Total content of a particular food item is taken as weighted sum of the food item and %V as the percentage composition.

The % IRI is expressed as %IRI= (IRI/ Σ IRI)x 100. This modified formula of IRI is based on the reason that it is realistic to base assessment of dietary importance upon unrelated methods[3], and that the indices combining values from different sources are more representative[14]. Data on non-target benthic fauna collected from the trawl grounds were also analyzed.

Results and Discussion

All the three species of fishes selected for the present study constitute important components of the demersal fishery resource of India in general and that of west coast in particular[15-23]. Their importance in the fish catch from both Vizhinjam and Neendakara has also been reported by fishery biologists[15,16,20]. At Vizhinjam, they are caught by non-mechanised and mechanised boats using conventional fishing gears like konchuvala, boat seine, hooks and line, drift net etc., while at Neendakara the main fishing gear used is trawl net. On many occasions they constitute by-catch of prawns from trawling boats of different sizes.

Of the three species selected for the present study, the biological aspects, especially food and feeding habits of *Nemipterus japonicus* have been well documented[24-32]. However, only limited information exists regarding *Priacanthus hamrur* and *Nibea maculata*[23,29]. Observations made earlier by many authors have revealed that all the three fishes are predatory carnivores and bottom feeders[22,23,27,31]. Food of these fishes, consists of crustaceans like squilla, prawns, mysids and other

Table 1 - Table showing values of Diet Overlap among the three species of fishes collected during the three seasons

	Pre-monsoon			Monsoon			Post-monsoon			Yearly		
Vizhinjam	*Nm*	*Nj*	*Ph*	*Nm*	*Nj*	*Ph*	*Nm*	*Nj*	*Ph*	*Nm*	*Nj*	*Ph*
Nibea maculata (Nm)	-	0.478	0.522	-	0.723	0.593	-	0.542	0.413	-	0 591	0 558
Nemipterus japonicus (Nj)	-	-	0.564	-	-	0.68	-	-	0 488	-	-	0 652
Priacanthus hamrur (Ph)	-	-	-	-	-	-	-	-	-	-	-	-
Neendakara												
Nibea maculata (Nm)	-	0 615	0.667	-	0 659	0.562	-	0 834	0.704	-	0 724	0 688
Nemipterus japonicus (Nj)	-	-	0 639	-	-	0.721	-	-	0 738	-	-	0.756
Priacanthus hamrur (Ph)	-	-	-	-	-	-	-	-	-	-	-	-

groups like squids, teleostean fishes, lamellibranch molluscs, annelids etc. with a preference for prawns and crabs.

The data pertaining to monthly variations in the feeding intensity, feeding index and the gastrosomatic index values were studied. These values also showed the similar trends in the feeding habits. The study indicated a positive relationship between feeding intensity and gastrosomatic index. The GaSI values indicate that the feeding conditions at Neendakara are better than that at Vizhinjam, except in the case of *Nibea maculata* during monsoon season. The condition coefficient was generally high in all the three fishes during the monsoon season. The condition indices are generally comparable for the three fishes at the two sites.

It may be noted that the diet overlap (Schoener index) that exist within the three fish species from Neendakara is appreciably larger than that observed at Vizhinjam (Table 1). The Schoener index of diet overlap assumes no competition (interaction) among the participating fish species for various food items. Resource availability is assumed to be unlimited, and this can generally be taken to be true in the case of the two

Table 2 - Table showing dietary similarity indices of the three fishes from the two sites

Fish species	Pre-monsoon	Mon-soon	Post-monsoon	Yearly
Nibea maculata	0 37	0.40	0.55	0.52
Nemipterus japonicus	0.57	0 54	0.39	0 60
Priacanthus harmur	0.57	057	0.47	0.63

sites considered here. Schoener index is treated as adequate to explain overlap values in the absence of resource availability data[11]. This index reflects sharing of major food categories among the fish groups. Comparing the values in the table, it may be observed that the overlap values at Neendakara are generally higher than the corresponding values at Vizhinjam. At Vizhinjam, comparatively moderate overlap exists between the three fish groups during the monsoon season (60% to 80%) whereas at Neendakara, the overlap is more pronounced during the post-monsoon season (more than 70%). This indicates that the resources available are shared by the three species of fishes at Neendakara, in a wider scale than that at Vizhinjam. Dietary similarity of the three fishes from the two study sites is presented in Table 2. The values of similarity index show that the dietary composition of the fishes at the two sites differs significantly.

The diet overlap indices given by Levins and Horn are calculated with the assumption that each category of fish utilizes certain fraction of the food items available to another category of fish so as to effect competition for food among the fishes living in the same environment. Comparison of the values of Horns index for the two study sites (Table 3) shows that the three fish species at Neendakara share the resources among themselves on a wider scale than the fishes at Vizhinjam. Comparison of the diet breadth data of the three fishes at Vizhinjam and Neendakara (Table 4) does not show any clear trend. The Index of Relative Importance (IR1) of each food component in the diet was calculated and presented in Tables 5 , 6 and 7. Seasonal changes in the preferred food items of the three fish species were observed from the data.

Table 3 - Asymmetrical diet overlaps of the three fishes at Vizhinjam and Neendakara. (Based on Levins and Horn formula)

	Vizhinjam				Neendakara			
Index	Pre-monsoon	Monsoon	Post-monsoon	Yearly	Pre-monsoon	Monsoon	Post-monsoon	Yearly
$Levins_{NMG1}$	0.53	0.81	0.65	0.63	0.57	0.71	0.94	0.83
$Levins_{NJg1}$	0.97	0.92	0.83	0.89	0.74	1.11	1.01	0.93
$Horn_{NM,NJg1}$	0.69	0.86	0.73	0.74	0.64	0.87	0.97	0.87
$Levins_{NJg2}$	0.67	0.94	0.52	0.78	0.77	0.79	0.84	0.83
$Levins_{PRg2}$	0.89	0.80	0.66	0.82	0.96	1.07	1.00	1.08
$Horn_{NJ,PRg2}$	0.76	0.86	0.58	0.80	0.86	0.91	0.92	0.93
$Levins_{NMg3}$	1.03	0.79	0.74	0.80	1.00	1.25	1.00	1.07
$Levins_{PRg3}$	0.42	0.82	0.45	0.53	0.61	0.51	0.79	0.75
$Horn_{NM,PRg3}$	0.60	0.80	0.56	0.63	0.77	0.80	0.88	0.87

Table 4 - Table showing values of Diet Breadth of the three species of fishes collected during the three seasons

Vizhinjam	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Yearly**
Nibea maculata	3.66	5.29	3.32	4.37
Nemipterus japonicus	6.64	6.02	4.25	6.24
Priacanthus hamrur	8.84	5.11	5.44	6.62
Neendakara				
Nibea maculata	4.15	3.12	5.00	4.90
Nemipterus japonicus	5.42	4.88	5.39	5.48
Priacanthus hamrur	6.74	6.62	6.42	7.15

Results of the present study conform to the previous observations made by several authors[4,22,23,27]. In both Vizhinjam and Neendakara all the three species feed on almost similar items of food. The higher diversity of food items indicates the diversity of food items present in the benthic habitat of both the study sites. The present results also indicate that they are selective predators preferring larger animals like fishes, prawns, squilla, squids etc. According to the optimal foraging theory, predatory fishes prefer larger prey to obtain more energy with optimum effort[33-35]. It is evident that not much variations exist in the diversity of food items consumed by these fishes and their quantity during different seasons. Similar observations on *Nemipterus japonicus* and *Priacanthus hamrur* have been made during previous studies[22,23,31,36].

Since feeding habits of these fishes reported from different areas of west coast and east coast of India show similarities in the diet composition and preference, the variation in percentage composition of food items from Vizhinjam and Neendakara fishing areas found during the present study can be attributed to the difference in their abundance in these two areas which in turn may be due to the degree of fishing pressure on these animals in both places. The high intensity of trawling in Neendakara might be affecting the benthic animals due to trawling which may indirectly be influencing the food preference of these fishes. Among the three species of fishes also variations in percentage composition of food items and their rank in abundance could be noticed. While index of preponderance values were compared, the difference in the ranking of food items observed may also be due to the variations in the abundance of these bottom fauna. However, when there is intense predation, there may be more competition for specific food items within the same habitat/ environment.

Table 5 - Comparison of values of Index of Relative Importance in *Nibea maculata* collected from Vizhinjam and Neendakara

	Vizhinjam						Neendakara					
	IRI			% IRI			IRI			%IRI		
Nibea maculata	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	0.00	0.00	0.00	0.00	0.00	0.00	495.34	5101.79	0.00	2.00	14.17	0.00
Fish larvae	0.00	36.43	0.00	0.00	0.08	0.00	0.00	10.91	0.00	0.00	0.03	0.00
Fish fragmnt	1002.97	20628.32	7832.04	21.37	44.25	30.02	6006.08	6100.63	3761.73	24.31	16.95	23.29
Fish Eggs	0.00	1772.62	0.00	0.00	3.80	0.00	448.96	130.91	0.00	1.82	0.36	0.00
Harp copepods	0.00	517.13	412.42	0.00	1.11	1.58	5.42	9.69	0.00	0.02	0.03	0.00
Isopods	0.00	0.00	23.06	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Amphipods	0.00	272.91	0.00	0.00	0.59	0.00	13.55	205.75	0.00	0.05	0.57	0.00
Ostracods	0.00	0.00	0.00	0.00	0.00	0.00	5.42	74.23	0.00	0.02	0.21	0.00
Mysids	0.00	709.84	723.39	0.00	1.25	2.77	6.78	37.79	0.00	0.03	0.10	0.00
Euphausids	0.00	0.00	0.00	0.00	0.00	0.00	31.61	0.00	0.00	0.13	0.00	0.00
Prawn	136.72	18.87	3274.32	2.91	0.04	12.55	282.33	776.55	59.09	1.14	2.16	0.37
Crab-megalopa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crab	364.77	0.00	0.00	7.77	0.06	0.00	93.63	12.48	344.50	0.38	0.03	2.13
Squilla	0.00	0.00	0.00	0.00	0.00	0.00	40.66	0.00	118.18	0.16	0.00	0.73
Alimalarva	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lucifer	0.00	69.50	551.63	0.00	0.15	2.11	0.00	0.00	0.00	0.00	0.00	0.00
Cumaceans	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crustacean fragments	182.11	319.78	186.99	3.88	0.69	0.72	580.17	1142.05	1388.93	2.35	3.17	8.60
Sepia	0.00	0.00	0.00	0.00	0.00	0.00	18.08	0.00	0.00	0.07	0.00	0.00
Loligo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bibalves	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gastropods	0.00	3.14	0.00	0.00	0.01	0.00	5.42	47.39	0.00	0.02	0.13	0.00
Nematodes	0.00	0.00	473.16	0.00	0.05	1.81	234.08	132.83	0.00	0.95	0.37	0.00
Nereis	0.00	25.46	567.56	0.00	0.05	2.18	216.81	41.19	0.00	0.88	0.11	0.00
Digested matter	3007.27	22246.83	12040.70	64.07	47.72	46.15	16225.78	22177.96	10479.48	65.66	61.60	64.88
Unidentified	0.00	0.00	11.57	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00

Table 6 - Comparison of values Index of Relative Importance in *Nemipterus japonicus* collected from Vizhinjam and Neendakara.

	Vizhinjam						Neendakara					
Nemipterus japonicus	IRI			% IRI			IRI			%IRI		
	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	0.00	764.40	71.77	0.00	6.19	0.67	237.35	0.00	41.66	1.24	0.00	0.05
Fish larvae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.77	0.00	0.00	0.02	0.00
Fish fragmnt	644.32	2018.33	779.69	3.22	16.35	7.24	2396.86	5849.71	7251.78	12.49	14.60	9.14
Fish Eggs	148.69	87.21	75.57	0.74	0.71	0.70	525.13	59.55	58.33	2.74	0.15	0.07
Harp.copepods	0.00	0.00	7018	0.00	0.00	0.07	0.00	0.00	9.59	0.00	0.00	0.01
Isopods	0.00	0.00	0.00	0.00	0.00	0.00	5.51	43.94	5.21	0.03	0.11	0.01
Amphipods	0.00	713.79	0.00	0.00	5.78	0.00	8.24	5.54	0.00	0.04	0.01	0.00
Ostracods	0.00	54.95	0.00	0.00	0.45	0.00	84.06	35.60	37.41	0.44	0.09	0.05
Mysids	0.00	19.71	123.01	0.00	0.16	1.14	0.00	31.47	1626.99	0.00	0.08	2.05
Euphausids	0.00	0.00	0.00	0.00	0.00	0.00	26.37	0.00	0.00	0.14	0.00	0.00
Prawn	58.68	217.34	1600.43	0.29	1.76	14.86	34.94	2511.21	6654.02	0.18	6.27	8.39
Crab-megalopa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crab	226.54	234.75	0.00	1.13	1.90	0.00	13.19	925.94	1345.99	0.07	2.31	1.70
Squilla	1968.65	0.00	0.00	9.83	0.00	0.00	0.00	4.10	271.85	0.00	0.01	0.34
Alimalarv	0.00	18.94	0.00	0.00	0.15	0.00	0.00	0.00	2489.94	0.00	0.00	3.14
Lucifer	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cumaceans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crustacean fragments	6198.97	2744.08	1338.34	30.95	22.23	12.43	1165.63	5685.56	2568.23	6.07	14.19	3.24
Sepia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Loligo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bibalves	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gastropods	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nematodes	22.07	797.42	513.86	0.11	6.46	4.77	1528.51	2227.92	742.21	7.96	5.56	0.94
Nereis	0.00	0.00	41.05	0.00	0.00	0.38	578.96	102.09	0.00	3.02	0.25	0.00
Digested matter	1762.49	4675.37	6195.83	53.73	31.87	57.54	12586.17	22579.96	56263.85	65.58	56.35	70.88
Unidentified	0.00	0.00	20.49	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00

Table 7 - Comparison of values Index of Relative Importance in *Priacanthus hamrur* collected from Vizhinjam and Neendakara.

Priacanthus hamrur	Vizhinjam						Neendakara					
	IRI			% IRI			IRI			%IRI		
	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	551 87	1102 66	317 38	0 33	0 68	0 23	676 06	4506 59	2445 64	0 78	3 25	1 79
Fish larvae	0.00	0 00	0 00	0 00	0 00	0 00	0 00	45 23	71 88	0 00	0 03	0 05
Fish fragmnt	1358 45	1967 95	2707 87	0 82	1 22	1 95	3639 96	8526 60	11006 69	4 18	6 14	8 07
Fish Eggs	20146 05	13612 42	2086 13	12 21	8 42	1 50	6912 26	8070 49	9985 07	7 94	5 82	7 32
Harp copepods	0.00	6198 92	83 19	0 00	3 83	0 06	161 68	90 24	26 94	0 19	0 07	0 02
Isopods	129 59	5599 86	85 45	0 08	3 46	0 06	16 64	56 15	0 00	0 02	0 04	0 00
Amphipods	102.53	1306 57	101 16	0 06	0 81	0 07	728 36	1620 41	0 00	0 84	1 17	0 00
Ostracods	6 33	835 26	129 17	0 00	0 52	0 09	232 19	1155 05	1116 89	0 27	0 83	0 82
Mysids	175 56	3623 42	11029 28	0 11	2 24	7 94	23 48	4659 83	9889 05	0 03	3 36	7 25
Euphausids	43 09	243 15	788 13	0 03	0 15	0 57	5591 00	19 01	1023 89	6 42	0 01	0 75
Prawn	63496 43	20402 55	44842 17	38 50	12 62	32 27	840 39	25440 15	8320 40	0 97	18 33	6 1[illegible]
Crab-megalopa	0.00	785 41	33.48	0 00	0 49	0 02	84 33	407 41	20 48	0 10	0 29	0 00
Crab	885 49	4108 55	578 10	0 54	2 54	0 42	1130 52	165 99	577 20	1 30	0 12	0 42
Squilla	60 86	4036 25	0 00	0 04	2 5[illegible]	0 00	14 97	42 29	0 00	0 02	0 03	0 00
Alimalarva	0 00	1918 17	0 00	0 00	1 19	0 00	0 00	169 06	0 00	0 00	0 12	0 [illegible]
Lucifer	0.00	0.00	0 00	0 00	0 00	0 00	6024	1 84	0 00	0 01	0 00	0 00
Cumaceans	0.00	0 00	11 35	0.00	0 00	0 01	0 00	0 00	0 00	0 00	0 00	0 00
Crustacean fragments	24463.84	14179 70	8008 64	14 83	8 77	5 76	13213 64	10359 45	14007 87	15 18	7 46	10 27
Sepia	0 00	0 00	14.44	0 00	0 00	0 01	9.98	23 91	53 88	0 01	0 02	0 04
Loligo	0 00	0 00	0 00	0 00	0 00	0 00	4 99	0 00	0 00	0 01	0.00	0 00
Bibalves	0.00	0.00	34.75	0.00	0 00	0 03	0 00	[illegible]	0 00	0 00	0 00	0 00
Gastropods	107 21	62.78	40 31	0.07	0 04	0 03	19 96	2 45	23 06	0 02	0 00	0.00
Nematodes	987.99	292.3	0.00	0.60	0.18	0 00	96 18	[illegible]	2529 49	0 11	0 15	1 86
Nereis	3218.15	130.32	0 00	1.95	0.08	0 00	471.57	25 96	25 33	0 54	0 02	0 00
Digested matter	49119.14	81167.37	68029 01	29.78	50 20	48 96	53135 59	73168.85	75232 [illegible]	61 03	52.72	55.17
Unidentified	83 86	128.52	28 01	0.05	0.08	0 02	52 55	2.76	0.00	0 06	0 00	0.00

Results of the estimation of feeding intensity of the fishes and their comparison between the two sites also indicated a noticeable difference. This difference in feeding intensity is an indication regarding the availability or nonavailability of preferred food items in the environment[36]. Comparison of the feeding index showed high intensity in Vizhinjam compared to Neendakara. Similarly the higher percentage of empty stomachs in samples collected from Neendakara is an indication of shortage of the most preferred food item in the environment, especially prawns. Trawling may be depleting the prawn resource of Neendakara area and hence samples of the fishes collected from this area may naturally be having shortage of that item in the environment. This is being reflected in the feeding intensity also. Results on gastrosomatic index and the condition factor of fishes support this view. Further, it is noticed that though, the three fishes share almost the same prey items; there exists variations in the similarity index among the three species and also among the different seasons.

The diet overlap values (Schoener index) help in assessing the competition among species and also in comparing different habitats without information on resource availability[11]. The results show that the resources available are shared by the three species in a more wider scale at Neendakara than at Vizhinjam. Diet overlap values also indicate a decrease in the food resource at Neendakara than at Vizhinjam. This change in predation pattern may again be due to the high fishing pressure on the environment directly and indirectly. A decrease in the preferred items like prawns, crabs etc. might definitely be adversely affecting the food preference and predatory efficiency in that habitat. The fishes are forced to share the available food items in a wider scale at Neendakara than at Vizhinjam. Wh . the benthic animals are sparse, diet overlap has been found to be low or in other words, different species will be exploiting different prey items[37]. Indices of relative importance of different food items during the three seasons helped in assessing the shift in predatory pattern of the three species during the different seasons. In the post-monsoon season a different dietary pattern was observed probably due to the closed season and subsequent stabilization of the bottom fauna.

A comparison of these indices clearly indicates that Neendakara is a highly disturbed area, which affects the food resources of the species studied. In Neendakara, these predatory fishes have to forage on more varieties of food items, probably due to the shortage of the preferred items. Nevertheless, they have to search for more prey items and also to spend more energy in foraging on more varieties of prey items The comparatively higher diversity values observed at Neendakara and higher diet breadth supports this view.

Otter trawling and its effect on the sea bed have been studied in detail as early as in 1970[38]. The impact on sea bed, especially trawl tracks has been reported to be 8 - 17 cm in mud and 0-5cm in sand[39]. On muddy fine sand the impact is about 10-15 cm. Moreover, the duration of the trawl tracks will be very short due to the pattern of currents. The important effect due to the tracks is sediment re-suspension and subsequent dislocation of benthic fauna due to the turbulence created by the net. This post trawling stress may also result in habitat modification, which in turn may reduce habitat complexity, resulting in fluctuations within benthic communities[40,41]. These fluctuations are reflected in the predatory behaviour and food partitioning of demersal fishes. Studies by Messieh *et al* [42] have shown incidental mortality and increased susceptibility to predation of benthic animals and also alteration of chemistry and texture of the sediments. Reduction in species diversity, change in relative abundance of species etc. are also potential effects of trawling.

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Detection of *Vibrio* proteins from diseased *Penaeus monodon (Fab.)* by ELISA and Western blotting

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Abstract

Vibrio species were isolated from black spot diseased *Penaeus monodon* Proteins were isolated from bacteria and subjected to Enzyme Linked Immuno Sorbent Assay (ELISA). The pathogenic protein was blotted by Western blotting. SDS-PAGE analysis revealed the presence of 29 kDa protein unique to pathogenic condition The antigens were detected by ELISA

Key words: shrimp, ELISA, SDS-PAGE, blotting.

सारांश

ब्लैक स्पाट रोग से पीडित *पीनियस मोनोडान* के शरीर से *वाइब्रियो* स्पीसीज को प्रथक्करित किया गया। उक्त बैक्टीरिया से प्रथक्करित प्रोटीन का एलीसा तकनीक द्वारा अध्ययन किया गया। इसके हानिकारक प्रोटीन को वेस्टर्न ब्लाटिंग द्वारा सशक्त किया गया। एस डी एस -पेज प्रक्रिया द्वारा इसमें ऐसे विशिष्ट 29 kDa प्रोटीन मिले जो हानिकारक व्यवस्था से सबद्ध थे। एलीसा तकनीक द्वारा इसके एन्टीजेन पहचाने गये।

सांकेतिक शब्द : झीगा, एलीसा, एस०डी०एस०–पेज, ब्लाटिंग

Introduction

The prawn/shrimp industry is facing serious problem with several types of diseases caused by bacteria, fungi and viruses. Bacteria of the genus *Vibrio* are ubiquitous in the marine and estuarine aquatic ecosystem in which shrimp occur naturally and are farmed[1]. All life stages of penaeid shrimp may be affected[2]. Antigen can be detected or measured by Enzyme Linked Immuno Sorbent Assay (ELISA)[3] in which the antibody is immobilized. A sample containing antigen is added and allowed to react. A second enzyme linked antibody, specific for different epitope on the antigen is added. After adding the substrate the colored reaction product is measured. The protein mixture is electrophoresed, the protein bands are transferred to a nitrocellulose membrane with enzyme linked polyclonal or monoclonal antibody specific for the protein of interest[4]. Hence an attempt has been made to detect the *Vibrio* protein by ELISA and Western blotting.

Material and Methods

The diseased prawn showing black spots were collected from culture ponds of Mallipattinum, Tamil Nadu, India. Swab was plated on Thiosulphate Citrate Bile salt Sucrose (HiMedia, Mumbai) (TCBS>pH8.5 ± 0.2) agar,incubated for 24hrs at 37^0C. Colour and morphology of the colony were recorded and subjected to various biochemical tests.[5] By comparing the results of biochemical tests with Bergey's Manual of Determinative Bacteriology[6] and a set of keys for biochemical identification of environmental *Vibrio sp*[7], the isolates were characterized. The total protein of diseased and control *P.monodon* and isolates of *Vibrio sp* were estimated by Biuret method[8]. The protein were

Table 1 - Biochemical Characteristics of *Vibrio spp.*

S. No		Characteristics		
		V parahae-molyticus	*V. orientalis*	*V mediterr-anei*
1.	Colour of the colony on TCBS Agar	Green	Yellow	Yellow
2.	Colony morphology	Smooth	Granular	Mucus
3.	Gram's staining	-ve Rod	-ve Rod	-ve Rod
4.	Size of the colony (mm)	3	3	3
5.	Cell Size (Length - mm)	0.8	0.8	2.56
6	Diameter	0 3	0.3	6.1
7.	Motility	+	+	+
8.	Indole	-1	4-	4-
9.	Methyl Red	-	-	+
10.	Voges Proskauer	-	-	-
11.	Citrate	+	+	+
12.	Catalase	+	+	+
13.	Oxidase	+	+	+
14.	Urea hydrolysis	+	+	+
15.	Dextrose	A	A	A
16.	Sucrose	A	A	A
17.	Lactose	-	-	A
18	Maltose	AG	A	A
19.	Mannitol	A	A	+
20.	Arabinose	+	A	A
21.	Growth in 0% NaCl	+	+	+
22	Growth in 1%NaCl	-	+	-
23.	Growth in 6% NaCl	+	+	+
24.	Growth in 8% NaCl	-	-	+
25.	Growth at 4°C	-	-	-
26.	Growth at 30°C	+	+	+
27.	Growth at 40°C	-	-	-
28.	Growth in pH4	+	+	-
29.	Growth in pH9	+	+	+
30.	Gelatin hydrolysis	-	-	+
31.	Starch hydrolysis	-	+	+
32.	Ciprofloaxin sensitivity	+	+	+
33.	Luminescence	-	-	-

Note : A - Acid, AG - Acid and Gas, + - Presence of growth, - - Absence of growth

Table 2 - Total Protein of Carapace and *Vibrio* Isolates.

Sample/Isolate	Replicates	%	X ± SD
	1	12.4	
Control Sample	2	12.6	12 566 ± 0.152
	3	12.7	
	1	12.09	
Infected Tissue	2	12.11	12.10 ± 0 01
	3	12 10	
	1	10.7	
V. cholerae	2	10.5	10 466 ± 0.251
	3	10.2	
	1	11.0	
V diazotrophicus	2	11.2	11 166 ± 0.153
	3	11.3	
	1	12.1	
V harveyi	2	12.2	12.3 ± 0.264
	3	12.6	

Fig. 1 - Total protein of Tergum and *Vibrio* isolates
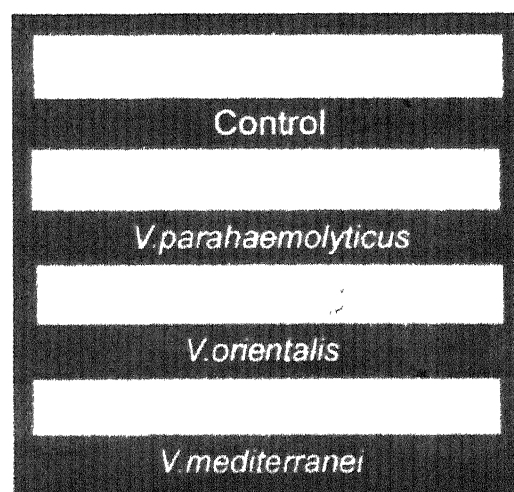
Fig. 2-A - Appearance of Blue spot
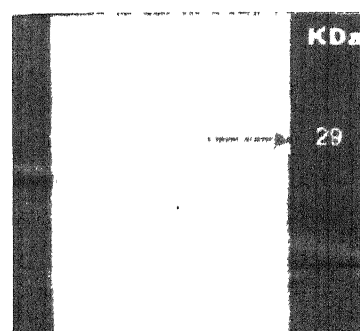
Fig 2-B - Blotting of 29 Kda protein band on Nc membrane

separated by Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (KT#30, GENEI, BANGALORE). The gel was removed and stained with Ezee blue for 1hr. After destaining the protein band was subjected to electroblotting[9] over nitrocellulose membrane (NC). The NC membrane was placed in blocking buffer (O N at 4^0C) V immersed in primary antibody and transferred to labeled secondary antibody followed by the addition of substrate (10 mins). The blot was removed, washed and air dried. DOT ELISA was performed[10] (GENEI BANGALORE).

Results and Discussion

The diseased prawn showed black spot on the tergum, on TCBS agar green and yellow colour colonies were observed. The results of biochemical tests revealed the presence of *V. parahaemolyticus*, *V. orientalis* and *V. mediterranei* (Table 1). The total protein showed significant variation ($P<0.05$; Table 2 and Fig 1). The DOT-ELISA revealed the presence of blue spot (Fig 2A). SDS-PAGE analysis revealed the presence of 29 kDa in *V. orientalis* and *V mediterranei* and 38 kDa protein in *V. parahaemolyticus* Western blot analysis showed the transfer of 29 kDa protein on NC membrane and was intensively stained (Fig 2B). Black spot was observed on the tergum[11]. The bacterial profile of water from the farms on the West Coast revealed that *V parahaemolyticus* ranged from 2.8% to 26% and were negative for haemolytic activity on Wagatsuma agar[12]. The serotype specific polyclonal antibodies to *V. cholerae* 0139 were usually prepared by repeated immunization of rabbits over an extended period of time by using *V cholerae* whole cells as the immunogen[13]. Tamil selvan[14] observed similar results in the presence of blue spots using *V. cholerae* whole cells as the immunogen.

SDS-PAGE and Western blot analysis carried out in the present study revealed the presence of protein band corresponding to 29 kDa. Manikandan[15] observed 29 kDa protein unique to the pathogenic strains of Leptospira.

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Differential Giemsa staining patterns in chromosomes of three Iranian species of *Fritillaria* spp. group

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Abstract

On the basis of C-band patterns and other karyologic features three species are described for the Iranian *Fritillaria* spp. group, *F caucasica* (Adams), *F uva-vulpis* (Rix) and *F assyriaca* (Baker) All the three species have a similar basic karyotype (n=12), consisting of large symmetric (m, sm) and smaller asymmetric (st, t) chromosomes, but C-bands differ between them. The bands are located at centromeric, intercalary, and telomeric positions and adjacent to secondary constrictions. *F caucasica* and *F assyriaca* differ from others in the possession of a submetacentric chromosome pair Further aspects of constitutive heterochromatin, heteromorphism, equilocal C-band distribution, C-banding evolution, and the role of C-banding in taxonomy are discussed.

Key words *Fritillaria species group*, C-banding, karyotypes, heterochromatin

सारांश

ईरान की फ्रिटिलेरिया स्पीसीज समूह की तीन प्रजातियो, *फ्रिटिलेरिया काकेसिया* (एडेम्स), *फ्रिटिलेरिया उवा–वूल्पिस* (रिक्स) ओर *फ्रिटीलेरिया एसीरियाका* (बेकर) के सी–पट्टो के नमूनो तथा दूसरे गुणसूत्री लक्षणो का वर्णन किया गया है। इन सभी प्रजातियो के मूल गुण–सूत्र (n=12) एक जेसे है, जिनमे बडे सुडौल तथा छोटे बेडोल गुण सूत्र है परन्तु इनके बीच के सी–पट्टे विभिन्नता दर्शाते है। ये पट्टे सेन्ट्रोमेरिक, अतर्वेशी तथा टिलोमेरिक स्थानो पर अनुपूरक सिलवटो के सन्निकट स्थित हैं। *फ्रिटीलेरिया काकेसिया* तथा *फ्रिटीलेरिया एसीरियाका* दूसरो से एक सब मेटासेन्ट्रिक गुणसूत्री जोडे की उपस्थिति की विभिन्नता दर्शाते हैं। इसके अतिरिक्त मूलभूत हेट्रोक्रोमेटिन, हेट्रोमार्फिज्म, समस्थानिक सी–पट्टो का वितरण, सी–पट्टो का विकासक्रम तथा सी–पट्टो की वर्गीकरण मे भूमिका का भी पर्यालोचन किया गया है।

सांकेतिक शब्द : *फ्रिटिलेरिया स्पीसीज समूह*, सी–पट्टी, मूलगुण सूत्र, हेट्रोक्रोमैटिन

Introduction

As part of a larger research programme on the systematics and evolution of liliaceous plant groups of South West Asia and the Mediterranean, the author previously studied the Giemsa C-banded karyotypes in Iranian species of *Rhinopetalum* and *Fritillaria* subgen. *Petilium* and *Theresia* (Bakhshi Khaniki 1998)[1]. This study clearly demonstrated that Giemsa C-banding patterns contribute significantly to infrasubgeneric grouping and generic demarcation, and that the so-called "banding style" may cast new light on evolutionary relationships both within and between the groups.

Rix[2] and Rechinger[3] have classified all Iranian species of *Fritillaria*[4] in four subgenera: *Fritillaria, Theresia, Petilium* and *Rhinopetalum,* of which *Rhinopetalum* is now accepted as a distinct genus. *Fritillaria* is morphologically divided into three species, *F. crassifolia, F. kotschyana* and *F. caucasica. F. crassifolia* and *F. kotschyana* show very similar flowers and leaf morphology. They can be divided into two complexes based on nectary characters[4]. *F. caucasia* shows comparatively small and narrowly campanulate flowers, and the nectaries are usually placed either at the base or only 0.5-1 (-2) mm above the base of the perianth.

Earlier studies of the distribution of constitutive heterochromatin in chromosomes of some Iranian species belonging to genus *Fritillaria* showed that the incidence and pattern of C-bands in this group may throw some light on evolution and speciation within the genus[5,6]. Unfortunately the whole material stemmed from a horticultural collection with no information regarding exact origin or number of populations.

The aim of the present investigation is to give a general over view of the variations of C-banding patterns found in the Iranian *Fritillaria* spp. group to provide data useful for phylogenetic and cytotaxonomic works.

Material and Methods

The specimens for this study were collected by the author from different localities in Iran (Table 1),

except *F. uva-vulpis* which was received from some other source. The bulbs collected were cultivated under uniform conditions in clay pots in indoor beds, in order to check the morphological characters and also for root tip preparations for chromosomal studies. Each population was assigned a collection number preceded by the abbreviation GBK which stands for the author's name. For chromosome studies, root tip meristem was used. The roots were treated with a mixture of 0.2% colchicine and 0.002 M 8-hydroxyquinoline (1 vol. each) for 2-3 hours at room temperature, fixed in Carnoy,s fixative (ethanol/acetic acid 3:1) for 24 hours, transferred to 70% alcohol and stored at -20°C until used. Fixed material was washed in distilled water and hydrolysed in 1 M HCl for 15 min. at room temperature. After another rinse, they were softened in a mixture of 10%-20% pectinase (Sigma) dissolved in 40% glycerol and 1% cellulase (Calbiochem) (1 vol. each) at 37°C for 1 hour. For acetic orcein staining, only 5% powdered pectinase for 2-3 hours at room temperature was used.

The meristematic tip of the root was separated and squashed in 45% acetic acid on a clean glass slide. Coverslips were removed after freezing in liquid nitrogen and slides immersed in 95% ethanol, dried in compressed air and stored in an exsiccator for a week. Dried slides were treated in freshly prepared 5% Barium hydroxide [Ba(OH)2] at 50°C-52°C for 7 min., washed in running tap water for 1 hour and immersed in 2 x SSC (pH=7.0) at 60°C for 60 min. After a rinse in Sϕrensen's phosphate buffer (pH 6.81), cells were stained in 1.5% Giemsa (buffered to pH 6.81) for 5-8 min., rinsed, dried, and mounted in D.P.X. Karyograms were prepared from drawings of metaphase chromosomes by using camera lucida.

Karyotypes were characterized by 1) pairing[7] i.e. indicating the pairs of homologous chromosomes in decreasing order of the length 2) typifying the kind of chromosome, according to centromere position 3) ratio of long arm to short arm (L:S)[6, 7, 8, 9, 22]. All measurements and percentage values given in the tables are mean values from 10 plates from a representative population.

Results

The somatic chromosome number (2n) was 24 for all species studied. All of the populations listed in Table 1 were found to have C-bands in their chromosome complements, some more than the others. The banding patterns are presented in the form of idiograms (Fig. 2). Based upon the intensity of the stain and the constancy of the banding, these bands were divided into two main categories, namely major and minor bands. The major bands (solid black and stippled areas in idiograms) stained very darkly, could be observed in every cell and were apparent from prophase to the highly contracted metaphase chromosomes. The minor bands comprised two classes: 1) lightly stained bands (broken lines in the idiograms) which varied in intensity and appearance from cell to cell, and 2) dot-like bands (double dots in idiograms) which were irregularly present and mostly located at intercalary positions. The bands are further divided into four groups according to their position: centromeric bands situated at one or both sides of the centromere, intercalary bands, telomeric bands (at the chromosome ends), and bands adjacent to the secondary constriction in the short arm of the satellited chromosomes. Apart from these, in some species the short arms of some chromosomes are entirely heterochromatic. Summary of karyotype characters of the species studied are listed in Table 5 and the percentage of heterochromatin in the haploid complement of the species studied is presented in the Tables 5 and 6.

(1) *F. caucasica* Adams. A Giemsa C-banded plate is shown in Fig. 1 a. Some important particulars of the karyotype are listed in Table 2, The karyotype consists of two metacentric, one submetacentric, four subtelocentric and five telocentric chromosome pairs (Fig. 2a). The pair no. 3 was found to have a small satellite in the short arm. The Giemsa C-banding pattern is characterized by the following: Five centromeric bands exist in the short arms of chromosome pairs nos. 6, 8, and 10, and in the long arm of pair nos. 7 and 11; major intercalary bands are absent in the short arms, but present in the long arms of pair nos. 1, 3, 5, 9, 10 and 12; telomeric bands occur in the short arms of pair nos. 2 and 4, and in the long arm of pair no. 1; a band occurs adjacent to the secondary constriction in the short arm of pair no. 3. Apart from these, there are many minor bands irregularly distributed along the chromosome arms. Heteromorphy in band size was evident in the long arms of pairs nos. 1, 3 and 7. The karyotype formula is 2n=24=4m + 2sm +8st + 10t.

(2) *F. uva-vulpis* Rix. A Giemsa C-banded plate is shown in Fig. 1b. Some particulars of the karyotype are listed in Table 3. The karyotype contains two
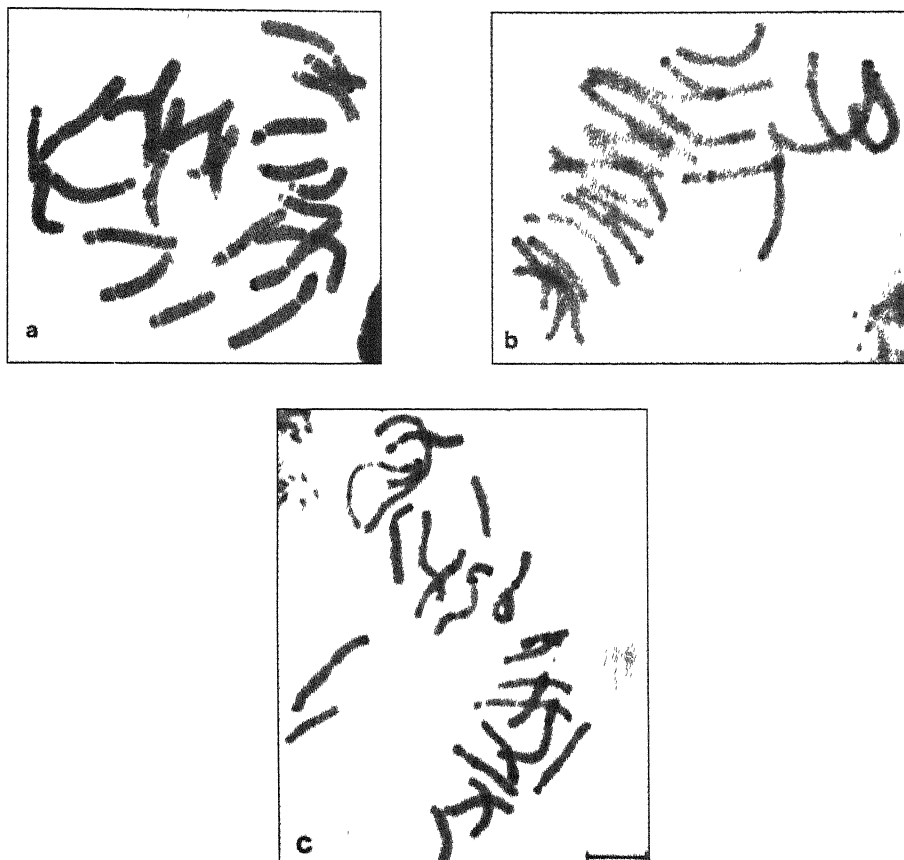
Fig. 1 - Giemsa C-banded metaphase of the species studied belong to *Fritillaria* group, a *F caucasica* (2n=24), Population GBK 70; b *F. uva-vulpis* (2n-24), Population TUBERGEN from Iraq; c *F. assyriaca* (2n-24), Population SØNDERHOUSEN 1106 from NE Turkey. - Scale: 10 µm.
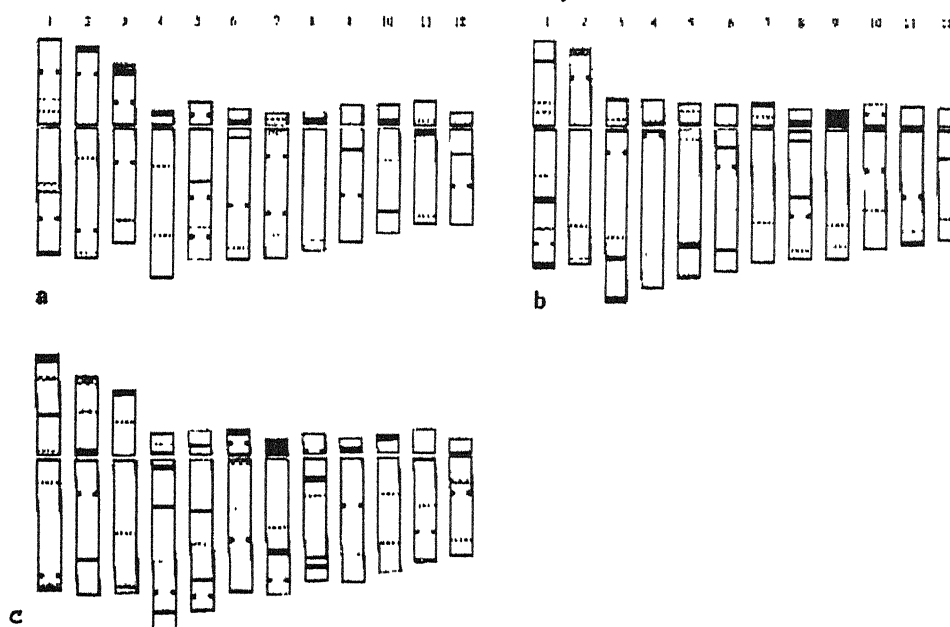
Fig. 2 - Haploid idiograms of the species studied belonging to *Fritillaria*. group, showing interspecific variation in karyotype. band size and numbers, a *F. caucasica*, b *F. uva-vulpis*, c *F. assyriaca*. Stipple areas: heteromorphic bands; double dots: dot-like "bands"; broken lines: thin pale bands. - Scale: 10 µm.

metacentric, eight subtelocentric and two telocentric chromosome pairs (Fig. 2b). Unlike *F. caucasica,* a pair of submetacentric chromosomes is absent. Of major C-bands there are eight centromeric bands present, five of which occur in the short arms of pairs nos. 4–5, 8 – 9 and 10, and three in the long arms of pairs nos. 3-4 and 8; distinct intercalary bands exist in the short arm of pair no. 1, and in the long arms of pairs nos. 1, 3, 5-6, 8 and 12; telomeric bands occur in the short arms of pairs nos. 2—3, 7, 9 (the short arm of pair no. 9 was entirely heterochromatic), and in the long arms of pairs nos. 1, 3, 5 and 11 no secondary constrictions were found. Apart from these and in accordance with the other species, chromosomes of *F. uva-vulpis* have some additional thin, pale and dot-like bands at intercalary positions. Band heteromorphy with respect to size was observed in chromosome pair nos. 1-2 and 5. The karyotype formula is 2n=24=4m+16st + 4t.

(3) *F. assyriaca* Baker. A Giemsa C-banded plate is shown in Fig. 1c. Some important particulars of the karyotype are listed in Table 4. The karyotype consists of two metacentric, one submetacentric, five subtelocentric and four telocentric chromosome pairs (Fig. 2c). Distinct centromeric C-bands are seen in the short arms of pairs nos. 2, 4, 7, and 9, and in the long arms of pairs nos. 6 and 11; major intercalary bands occur in the short arms of pairs nos. 1-2, 5, and in the long arms of pairs nos. 2-5, 7-8 and 12; telomeric bands are present in the short arms of pairs nos. 1-3, 6, and 10, and in the long arms of pairs nos. 1, 6, and 11; the short arm of the SAT-chromosome pair no. 7 was, except for the satellite, entirely heterochromatic. In addition to these major bands, there are some minor bands in the karyotype. Heteromorphy with respect to band size was evident in the chromosome pairs nos. 1-3, 6 and 12. A pair of submetacentric chromosomes is present in *F. assyriaca* as in case of *F. caucasica.* The karyotype formula is 2n=24=4m + 2sm +10st + 8t.

Discussion

The present observations show that three species of *Fritillaria* present C-bands, some more than the others (Table 5, 6). The chromosome number determinations of 2n=24 for *F. caucasica* and *F. assyriaca* are new reports for the geographic zones from which these plants were collected. However, they agree with determinations based on material of these taxa from other areas[5, 6]. The somatic chromosome number of 2n=24 for *F. uva-vulpis* is reported here for the first time.

In general, it seems that the amount of constitutive heterochromatin is relatively higher in species belonging to the subgenus *Fritillaria* than those of the subgenera *Petilium* and *Theresia*[5] Also, La Cour[5, 6, 8] believed that Old World species of *Fritillaria* usually have less heterochromatin than those of New World origin. One point perhaps worthy of consideration, particularly since there is little interspecific hybridization in fritillaries, is that the American fritillaries, with an abundance of heterochromatin, may possibly be relics. The forest or forest border habitats in north-west California where most of the American fritillaries occur have many tertiary relics. The climate of the area has been favourable for long-time survival, and the rocky serpentine type of terrain may have enabled some to survive free of competition. The evaluation of these findings is, however, a matter for the future, as the understanding of the evolution of heterochromatin is in many ways, far from complete.

There seems to be little doubt from the present observations that at least two classes of heterochromatin occur in *Fritillaria.* These are one represented by intensely stained and relatively thick (major) bands, the other by weakly stained, thin C-bands and/or dot-like bands (minor bands). Both classes have been previously recorded in *F. crassifolia*[5, 6, 9]. It is assumed that the pale bands and the dot-like bands are part of one and the same phenomenon and one which is most simply explained in terms of packing of the chromatin involved. Accordingly, it is believed that the two classes of hetcrochromatin diverge in respect to their degree of condensation. It may well be, as the present observations of interphase, and also previous findings in *Fritillaria* suggest, that the distinction exists for the whole of the mitotic cycle. The position of such bands in metaphase chromosomes has been demonstrated to correspond exactly with that of H-segments resolved as weakly stained regions in Feulgen squashes of roots from plants grown at low temperatures[10]. In a comparative study of *Frilillaria* and *Scilla,* the findings in *Fritillaria* were in marked contrast to those obtained in roots of *Scilla sibirica*[11, 12]. The H-segments of this plant could not be identified by the same criteria, although it is well-known that C-bands are demonstrable in its chromosomes with the Giemsa technique. Thus even at the time of these

Table 1 - Original sources of material studied of *Fritillaria* spp group.

Pop. no.	species	Locality
GBK 70	*F. caucasica*	Iran: E. Azarbaijan, road from Tabriz to Ahar, steep-stony slopes, 1800-2150 m
TUBERGEN s.n	*F. uva-vulpis**	Iraq: Rowandooz, Haji Omran.
SØNDERHOUSEN	*F. assyriaca**	Turkey: Agri, Tahır Pa. pass.

* Material for karyological studies obtained from cultivated plants in the Goteborg Botanical Garden, Sweden.

Table 2 - Details of karyotype in *F. caucasica* (GBK 70).

Pair	L+S(µm)	L(µm)	S(µm)	L+S%	L%	S%	L/S	Type
1	18.18	10.83	7.35	11.86	7.05	4.81	1.47	m
2	17.83	11 06	6 77	11 51	7 14	4 37	1.63	m
3	14.41	9.74	4 67	9.29	6.28	3.01	2.08	sm
4	13.88	12.72	1.16	8.96	8.21	0.75	10.94	t
5	13.23	11.29	1.94	8 54	7.29	1 25	5 83	st
6	12 45	11 16	1 29	8.03	7.20	0 83	8.67	t
7	12 07	11.09	0.98	7 78	7.15	0.63	11.34	t
8	11.42	10.44	0.98	7 37	6.74	0.63	10.69	t
9	11.34	9.66	1.68	7.31	6 23	1.08	5 76	st
10	10 76	8.93	1 74	6 88	5.76	1 12	5.14	st
11	10.12	8.10	2.02	6.53	5.23	1 30	4.02	st
12	9 21	8.18	1 03	5.94	5.27	0 67	7.86	t

studies, it was apparent that more than one class of heterochromatin was present in chromosomes of plants, corresponding to different degrees of condensation. In three *Fritillaria* species presently studied, different numbers of dot-like bands could be recognized in the chromosomes. These were mostly intercalary and partly centromerically positioned. Small centromeric dots were first demonstrated by Eiberg[12] in Giemsa preparations of human chromosomes.

Just as in animals, there is extensive variation of heterochromatin in plants. Most of the work on heteromorphism in plants has been based on C-banding which shows many interesting features. In particular, it has been possible in some species to make correlations with the phenotype of the plants. A number of examples of heteromorphism of C-bands in plants could be mentioned. For example, the chromosomes of *Scilla sibirica* show extensive heteromorphism in their heterochromatin, which is both C-banded and intensely fluorescent with Hoechst 33258. In several species of *Allium*, Vosa[13, 14] also described a number of different classes of heterochromatin, according to whether or not they were C-banded, and whether they showed increased or decreased fluorescence or no differentiation with Quinacrine or Hoechst 33258. The heterochromatic segments varied in number and size, and were reported to replace or possibly to add to, normal euchromatic segments. Marks and Schweizer[15] described heteromorphism for both number and size of bands in *Anemone*, while Filion[16] found large differences in banding patterns between different cultivars of *Tulipa*. Bentzer and Landstrom[17] described a homologous pair of chromosomes in *Leopoldia comosa* which exhibited between three and eight C-bands, and which also varied in width and staining intensity. Heteromorphy in *Fritillaria* and *Rhinopetalum* was also previously reported[1, 5, 6]. In addition to polymorphism in general chromosome morphology in *Fritillaria*, just as in *Tulipa* there is evidently also heteromorphy of

Table3 - Details of karyotype in *F uva-vulpis* (TUBERGEN from Iraq)

Pair	L+S(µm)	L(µm)	S(µm)	L+S%	L%	S%	L/S	Type
1	19 28	12.01	7 27	11.28	7.02	4 26	1.65	m
2	18.16	11 58	6.58	10.55	6.73	3.82	1.76	m
3	17.13	14.80	2.33	9.95	8.60	1.35	6 37	st
4	15.83	13.63	2.20	9.20	7.92	1.28	6 18	st
5	14.67	12.80	1.87	8.52	7.44	1.08	6 88	st
6	14 01	12 20	1.81	8.14	7.09	1.05	6 75	st
7	13 38	11.42	1.96	7.78	6 64	1.14	5.82	st
8	12 57	11.16	1.41	7 31	6 49	0.82	7.91	t
9	12 39	11 06	1.33	7.20	6.43	0.77	8.35	t
10	12.07	10.26	1.81	7.01	5.96	1.05	5.67	st
11	11.51	9.92	1.59	6 72	5.76	0 96	6 00	st
12	10 89	9.43	1 46	6 33	5.48	0 85	6 44	st

Table 4 - Details of karyotype in *F assyriaca*(SϕNDERHOUSEN 1106).

Pair	L+S(µm)	L(µm)	S(µm)	L+S %	L%	S%	L/S	Type
1	19.82	11 22	8.60	11.79	6 67	5.12	1.30	m
2	18.30	11.55	6 75	10.82	6.83	3.99	1.71	m
3	16.95	11.42	5.53	10.02	6.75	3.27	2.06	sm
4	16 44	14.65	1.79	9.71	8.66	1.05	8 16	t
5	14.88	12 96	1.92	8 79	7.66	1 13	6 77	st
6	13.60	11.53	2.07	8.04	6.81	1 23	5 53	st
7	13 03	11.68	1 35	7.69	6.90	0.79	8.73	t
8	12.20	10.52	1 68	7.21	6.22	0.99	6 28	st
9	11.94	10 72	1.22	7 06	6.34	0 72	8 80	t
10	11 42	9 87	1.55	6.74	5 83	0.91	6.40	st
11	10.92	9.03	1.89	6 45	5.34	1.11	4.81	st
12	9.60	8.46	1.14	5 67	5 00	0.67	7.46	t

bands. In agreement with conditions in other related genera, three types of band heteromorphy were encountered in the present investigation. Firstly, a heteromorphy occurs in size of a particular band in a homologous pair of chromosomes. This was mostly common and very distinct above all in meta- and submetacentric chromosome pairs of all species examined (Fig. 2, stippled areas). Secondly, heteromorphy may manifest itself in the presence or apparent absence of a particular band.

Heterochromatic segments also varied in amount, number and position between different species. It is apparent from this study that wide differences exist between fritillaries in the distribution and amount of detectable heterochromatin contained in their chromo-

Table 5 - Summaryof karyotype characters of the *Fritillaria* species studied.

Species	Range of chromosome length (μm)	Total haploid karyotype length (μm)	Karyotype formula
F caucasica	9.21-18 18	154 20	2n-24=4m + 2sm+8st+ 10t
F uva-vulpis	10 89-19.28	171.89	2n=24=4m + 16st + 4t
F assyriaca	9.60-19.82	169.10	2n=24=4m + 2sm+ 10st + 8t

Table 6 - Proportion (in %) of Giemsa C-bands to chromosome length.

Note: dot-like and thin pale bands (presented by double dots and broken lines in idiograms) are not considered in this calculation

Species	Pop no.	Chromosome no. 1	2	3	4	5	6	7	8	9	10	11	12	% of heterochromatin in haploid complement
F. caucasica	GBK 70	3.0	1.8	3 0	1 7	0 3	1.8	1.3	1.7	1.0	2.3	1.7	0 4	3 80
F uva-vulpis	TUBERGEN	6 1	2.3	3 5	2.0	4.4	1.8	1 0	4 4	5 1	0.0	1 5	0.8	4.96
F assyriaca	SФNDERHOUSEN	6.7	6.6	3 0	4.7	2 0	3.9	5 9	4.2	2.3	1.5	1.4	1 5	6.70

somes. A summary of heteromorphy, and number and distribution of bands is presented in Table 7. Heteromorphism is likely to be very much involved in the variation in total DNA connected to specific evolution. The set of bands revealed by C-banding procedures usually consists of different forms of heterochromatin[18], and intraspecific variation in C-banding heteromorphy must be largely due to differences of amounts and kinds of constitutive heterochromatin coupled with variation of chromosome contraction.

It seems that C-band material and heterochromatin in general are relatively plastic nuclear components which may be subjected to considerable quantitative and qualitative changes during karyotype evolution. C-band negative (and generally euchromatic) chromosome segments, on the other hand, may exhibit higher degrees of conservation. This phenomenon is already well known for plants and has also become particularly evident in certain mammalian systems where specific staining reactions such as Q- or G-banding also allows for discrimination of other chromosome segments than C-bands. A study on species of the American rodent genus *Peromyscus* showed that karyotype differences between the two species *P. crinitus* (2n=48, 56 chromosome arms per diploid complement) and *P. boylei* (2n=48, 96 chromosome arms) are solely due to differences in C-band content: the second arms, which differ in number, are totally heterochromatic in both species whereas the G-banding patterns of the C-negative arms were identical (Arrighi *et al.*)[19]. According to La Cour[5,6] out of thirty-seven species of *Fritillaria*, examined twelve out of twenty-seven Old World species and seven out of nine New World species had detectable heterochromatin. It was concluded that New World species overall have an appreciably higher content of heterochromatin. So, it is reasonable to assume that the direction of evolution in *Fritillaria* is towards heterochromatin increase. It is also clear from the present investigation that wide differences exist between fritillaries in the distribution and amount of heterochromatin even between Old World species. What does this mean in terms of evolution? The answer may possibly lie in the directed evolution of DNA with highly repetitive sequences, the most likely component of constitutive heterochromatin. A correlation between highly repeated satellite DNA and C-banding positive heterochromatin was demonstrated in *Scilla siberica* (Timmis *et al.*)[20]. For highly reiterated simple sequence DNA, some models of evolution have been proposed and it was suggested that highly repeated DNA, such as satellite DNA, is evolving more rapidly than unique sequence DNA. If the above correlation between highly repeated DNA and C-banding is generally true also for plant chromosomes, it may be possible to draw analogous lines for the evolutionary trends of C-band material in plants.

A model which attempts to explain the concept of equilocality (Schweizer and Loidl)[21] assumes that telomeres (including centric regions in the aero-telocentric

chromosomes) are C-band initiation sites. These regions are thus presumptive sites where heterochromatin amplification tends to be initiated, and from which heterochromatin sequences are dispersed. Transposition may be coupled with the amplification process. Transpositional spread requires proximity and/or physical contact. All the potential contact zones which lie at a similar distance from the centromere (i.e. equilocal sites) are potential sites of heterochromatin transfer from telomere to intercalary locations during evolutionary time period. This model of band distribution predicts that karyotypes consisting of chromosomes with approximately similar arm length will tend to have telomeric bands rather than intercalary bands, and a karyotype consisting of members with different arm ratios, will also tend to possess intercalary bands in addition to telomeric bands. It is very clear from the present investigation, that there is great diversity of arm ratios (L/S in Tables 2-4) in the karyotype of the *Fritillaria* species examined, and not surprisingly, in addition to telomeric bands, there are also many intercalary bands in the chromosomes.

The C-banding pattern is the existence of one common, and ± similar model of banding in a group or groups of plants is often useful in taxonomy. Banding patterns can be used to study chromosomal changes that have occurred between related species. In many cases differences in banding patterns between related species are very small, although complete identity is comparatively rare. More often, some differences can be found even between closely related species. Changes in the amount of heterochromatin, demonstrated by C-banding, are quite common. Several C-banding studies have attempted to recognise ancestral genomes by heterochromatin markers, especially in allopolyploids[22]. As regards taxonomic applications of C-banding, Marks and Schweizer (1974)[15] were the first to demonstrate

Table 7 - Frequency and distribution of C-bands and heteromorphic bands in the chromosomes of material studied of *Fritillaria* spp. group. Numerator: number of positions with heteromorphic bands; denominator total number of positions with bands
Note. dot-like and thin pale bands (presented by double dots and broken lines in idiograms) are not considered in this table.

Species	Band position	Chromosome no.											
		1	2	3	4	5	6	7	8	9	10	11	12
F caucasica	Intercalary	2/2	0/0	1/1	0/0	0/1	0/1	0/0	0/0	0/1	0/1	0/0	0/1
	Centromeric	0/0	0/0	0/2	0/1	0/2	0/1	0/1	0/1	0/0	0/1	0/1	0/0
	Telomeric	0/1	0/1	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0
	Sec constriction	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0
F. uva-vulpis	Entirely	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	heterochromatic short arm Intercalary	2/4	0/0	0/1	0/0	0/1	0/2	0/0	0/2	0/0	0/0	0/0	0/1
	Centromeric	0/0	0/0	0/1	0/2	0/1	0/0	0/0	0/2	0/1	0/1	0/0	0/0
	Telomeric	0/1	1/1	0/2	0/0	0/1	0/0	0/1	0/0	0/1	0/0	0/1	0/0
	Sec. constriction	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
F assyriaca	Entirely	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0
	heterochromatic short arm Intercalary	1/2	1/2	1/1	0/3	0/3	0/0	0/1	0/3	0/0	0/0	0/0	1/1
	Centromeric	0/0	0/1	0/0	0/1	0/0	1/1	0/1	0/0	0/1	0/0	0/1	0/0
	Telomeric	1/2	1/1	0/1	0/0	0/0	0/2	0/0	0/0	0/0	0/1	0/1	0/0
	Sec. constriction	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0
	Entirely heterochromatic short arm	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0

the impact that banding of *Anemone* and *Hepalıca nobilis* would have similar significence in plant systematics. C-banded karyotypes of ± related taxa indicated a relatively high degree of karyomorphological differentiation in some woody species of *Liriodendron* (Magnoliaceae,) and the results were considered in karyosystematic comparisons[23]. On the basis of C-banding, *Artemisia* (Compositae, Anthemideae) is characterized as a genus with few intercalary C-bands in chromosomes[22] and this result was in agreement with the results of Schweizer and Ehrendorfer[24], who found this pattern to be common in the tribe Anthemideae. A comparison between the C-banded karyotypes of two species of *Festucopsis* (Poaceae, Triticeae) supported their systematic position in two genera[24, 25].

Surveys of bulbous genera such as *Leopoldia* (Bentzer)[26, 27, 28], *Allium* (Vosa)[13, 14], *Fritillaria* (La Cour)[1, 5, 6], *Tulipa* (Blakey and Vosa)[29], *Scilla* (Greilhuber)[10, 11] and *Lilium* (Smith *et al*)[30] have been given All these studies show that a large amount of variation in heterochromatin amount exists within each genus, and chromosome banding results usually correspond to existing taxonomic groupings, although sometimes some disagreements or within-group heterogeneities were noted.

In *Fritillaria* s.lat, according to Bakshi Khaniki[1] large differences exist in the amount and distribution of heterochromatin, not only between groups but also within them, the divergence being extreme in the American subgenus *Liliorhiza,* or within the *Fritillarıa* group. In the present investigation, the chromosome banding results, for *Fritillaria* subgen. *Petilium* and *Theresia* corresponded to existing morphologically based taxonomic grouping. In the genus *Rhinopetalum,* the closely related *R gibbosum* and *R arianum* present almost identical banding patterns. Presence of a centromeric band in the short arm of the second pair of m-chromosomes in *R. bucharicum* distinguishes it from the other two species. These C-banding results confirm the morphological grouping of these *Rhinopetalum* species into two subgroups, based on nectary morphology[4].

It is also evident from the present investigation that certain differences exist in the amount and distribution of C-bands between closely related species. There is much divergence in C-banding pattern between the Iranian species of *Fritillaria* spp. group, of which *F. caucasica* and *F. assyriaça* possess a pair of submetacentric chromosomes with telomeric and intercalary bands. Apart from this, a pair of SAT-chromosome was observed in both species. There are many intermediates between *F caucasıca* and other species, particularly *F assyrıaca,* in the group in NE Turkey. Both species are decidedly similar in some morphologic characters and may be considered as a variant of one species. Similar banding results may confirm this suggestion. *F chlorantha* presented a comparatively high amount of heterochromatin, mostly distributed at centromeric locations. Furthermore, the short arms of pairs nos. 5 and 11-12 were entirely heterochromatic. This specıes is morphologically similar to *F atrolıneata*[2] maınly in the colour of its flowers, but exhibits a different C-banding pattern. *F. zagrica* is morphologically unique by having the dark purple flowers adorned with a bright yellow apex. It was the only species in this group which presented accessory B-chromosomes[1]. It is evıdent here that large differences exist in banding patterns in species of the *Fritıllarıa* spp. group, and it is difficult to formulate a basic bandıng pattern to confirm the taxonomic grouping, though the latter is well documented by morphologic characters. The C-banding results for Iranian species belonging to the *Fritillaria* spp. group is in agreement with that of La Cour[5, 6, 8] on European species for the same group. Congruence of taxonomic grouping with similarity in chromosome structure is not expected to occur generally even in an ideally perfect system[1].

This survey has shown that C-band patterns can often be useful in deducing relationships between species belonging to *Fritillaria* spp. group, and that they are also useful for species separation.

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Studies on the diversity and pattern of vertical distribution of some epiphytic pteridophytes on their host plants of southern Sikkim, India

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Abstract

The objectives of the present work were to determine the epiphytic pteridophyte composition, pattern of vertical distribution and diversity of epiphytic pteridophytes on the host trees of Sikkim area as well as to demarcate the 'specialist species' (species having restricted vertical distribution). The occurrence of epiphytic pteridophytes in relation to the type of forest, altitude and environmental factors like rainfall, relative humidity and temperature have also been determined. It was found that the upper hill forest zone harbours the highest diversity of epiphytic pteridophytes The richness of epiphytic pteridophytes was maximum in the upper foot of the host plants, while it was minimum in the tree top region *Pyrrosia lanceolata* (L.) Farwell, *Oleandra wallichii* (Hook.) Pr, *Polypodioides lachnopus* (Wall. *ex* Hook) Ching and *Arthromeris walttchiana* (Spreng.) Ching were found to have wide vertical distributions. While, *Pseudodrynaria coronans* (Wall. *ex* Hook.) Ching *Microsorum punctatum* (L) Copel., *Nephrolepis cordifolia* (L.) Pr., *Lepisorous ussuriensis* (Regel *et* Maack) Ching, *Polypodiastrum argutum* (Wall. *ex* Hook) Ching, *Pyrrosia manii* (Gies.) Ching, *Selaginella involvens* (Sw.) Spring and *Ctenopteris subfalcata* Blume *ex* Kunze have restricted vertical distribution on the host plants. Specialist species vary with the altitude, except two ferns and the generalist species were more or less common in different altitudinal zones

Key words : vertical distribution, epiphyte diversity, specialist species, generalist species, conservation, Sikkim.

सारांश

सिक्किम के पोषी वृक्षो पर ऐसे टेरिडोफाइट पौधे, जो अधिपादप हो, की सरचना, खडी विस्तारिता तथा विभिन्नता का अध्ययन, साथ ही साथ विशेषज्ञ प्रजातियो (जो सीमित खडी विस्तारिता दर्शाती हो) का निर्धारण, वर्तमान कार्य का उद्देश्य है। इसके अतिरिक्त इस प्रकार के टेरिडोफाइट किस प्रकार के वनो मे पाये जाते है, कितनी ऊँचाई पर पाये जाते है तथा वातावरणीय कारको जैसे वर्षा, तुलनात्मक आद्रता तथा तापमान का इनके उपस्थिति पर क्या प्रभाव पडता है, इन सभी तथ्यो का भी अध्ययन किया गया है। अधिपादप प्रकृति के टेरिडोफाइट पौधो की अत्यधिक विभिन्नता ऊपरी पहाडी वन क्षेत्र मे मिली है। इस प्रकार के टेरिडोफाइट पौधो की अधिकतम प्रचुरता जहॉ पोषी पौधो के तने के ऊपरी हिस्से मे दिखती है, वही न्यूनतम प्रचुरता पेड के ऊपरी हिस्से मे दिखती है। *पाइरोसिया लैन्सियोलेटा* (लीनियस) फारवेल, *ओलीएन्ड्रा वालिची* (हुक) प्र०, *पालिपोडायोयडिस लैचनोपस* (वाल एक्स हुक) चिग और *आर्थोमेरिस वैलीचियाना* (स्प्रेग) चिग मे विस्तृत खडी विस्तारिता दिखती रही है, जबकि *सूडोड्राइनेरिया कोरोनैन्स* (वाल एक्स हुक) चिग, *माइक्रोसोरम पंकटेटम* (लीनियस) कोपेल, *नेफ्रोलेपिक कार्डीफोलिया* (लीनियस) प्र०, *लेपीसोरस असूरियेन्सिस* (रीगेल एट माक) चिग, *पालिपोडयास्ट्रम आर्गूटम* (वाल एक्स हुक) चिग *पाइरोसिया मैनाई* (गीज) चिग, *सिलैजीनेला इन्वाल्वेन्स* (स्यू) स्प्रिग तथा *टिनोप्टेरिस सबफैल्काटा* ब्लूम एक्स कुंज मे पोषी पौधो पर सीमित खडी विस्तारिता दिखती है। विशेषज्ञ प्रजातियॉ, दो पर्णागो को छोड कर ऊँचाई के अनुरूप विभिन्नता दर्शाती है। सामान्य प्रजातियॉ, विभिन्न ऊँचाइयो के क्षेत्रो मे लगभग एक सी है।

सांकेतिक शब्द : खडी विस्तारिता, अधिपादप विभिन्नता, विशेषज्ञ प्रजातियॉ, सामान्य प्रजातियॉ, सरक्षण, सिक्किम।

Introduction

The altitude of Sikkim varies from 224 m to 8500 m. The climate and vegetation vary with the change in altitude. Altitude-wise vegetation of this state is divided into five zones[1]. These are—

1. Low hill forest (up to 750 m).
2. Middle hill forests (750-1500m).
3. Upper hill forests (1500-2700 m).
4. Rhododendron-Conifer zone forests (2700-4300m).
5. High altitude scrub and grassland (3600-4300m)

Epiphytic pteridophyte diversity also varies with the change of climatic conditions and altitudinal ranges. In

pteridophytes, most of the members of the families of Lycopodiaceae, Vittariaceae, Hymenophyllaceae, Davalliaceae, Aspleniaceae and Polypodiaceae are adapted to epiphytic mode of life and they contribute a significant proportion to the pteridophytic flora particularly in Sikkim Himalaya. Epiphytes comprise about 10% of the total vascular plants of the world[2] but in Darjeeling and Sikkim Himalaya[3] it is about 20%.

Apart from traditional systematic classification, the epiphytic pteridophytes have been classified in several ways[3-9], mainly on the basis of nature of habitats and their associations. According to the vertical height sections, host trees are divided into four major regions[7-8], which are (I) foot region, (II) upper foot region, (III) branch region and (IV) tree top region. Epiphytic pteridophytes grow in different positions on the host trees. This pattern is known as vertical distribution[7]. In India, many workers studied the floristic composition of pteridophytes since the time of Beddome[10]. Many workers[11-16] also contributed to the understanding of ecology of epiphytes. Workers outside India[2, 7, 8, 17-20] contributed on the vertical distribution and ecology of vascular epiphytes, but very little work[21] has been done in India regarding the vertical distribution and diversity of epiphytic pteridophytes with special reference to Sikkim Himalayas. So, this study was undertaken with a view to determine:

a. The composition, pattern of vertical distribution and richness of epiphytic pteridophytes of southern Sikkim.

b. The specialist epiphytic species and generalist epiphytic species.

c. The diversity of epiphytic pteridophytes in different altitudinal zones.

d. Whether the vertical distribution and occurrence of epiphytic pteridophytes are related to the type of forest, altitude, environmental factors or other characteristics of trees that serve as host.

e. The epiphytic species rich areas of southern Sikkim, which could be recommended for *in situ* conservation for preserving biodiversity.

Material and Methods

During field survey to southern Sikkim for about 4 years, (2002-2006) several places were visited. Southern Sikkim is a large area comprising about 3500 sq km. The study area was divided into 6x6 km grid cells following Bolos and Romo's[22] method. As forest allow greater establishment and success of epiphyte vegetation[23], 28 grid cells of well dense forests, including wildlife sanctuaries, were chosen for study. Trees were selected randomly within a grid cell where epiphytic growth was present. They were subjected to fulfill the following parameters.

1. They are in good health.
2. They could be climbed.
3. They did not have exfoliating bark, because this did not allow establishment of stable epiphytic communities.

Using simple climbing equipments, the epiphytic pteridophytes were sampled on the host trees and the following data gathered:

1. Height of the host trees.
2. Kind of epiphytic species.
3. Height section of the host, from which the epiphytic species were collected.

Frequency of the epiphytic pteridophytes was calculated by using transect method from 28 grid cells.

Following Johansson[7], Cornelison and Steege[8] the host trees were divided into different height sections. Collections were identified with the help of authenticated specimens at Central National Herbarium (CAL) and published literatures[3, 10, 24-26]. The altitudinal classifications of vegetation given by Humboldt[1] and Champion and Seth[27] were followed. Species richness with different environmental and geographical factors was analyzed statistically.

Observations

Among the 28 grid cells of southern Sikkim studied for diversity and vertical distribution of epiphytic pteridophytes, different places having different frequency (f) of occurrences were noted. Frequencies of each epiphytic pteridophyte along the altitudinal zones are given in Table 1.

The low and middle hill forest zone (up to 1500 m) had only 20 epiphytic pteridophytic species distributed vertically in the first three regions of the host trees.

Table 1 - Frequency of occurrence of epiphytic pteridophytes in different altitudinai zones

Sl. No.	Sc. Name(s) of the plants	Frequency in Low and Middle hill forest zone (Upto 1500m) ±SD	Frequency in upper hill forest zone (1500-2700m) ±SD	Frequency in Rhododendron-Conifer zone (2700-3600m) ±SD
1.	*Asplenium ensiforme* Wall. *ex* Hook. & Grev	-	43.1±2 6	61.8±3 4
2	*Asplenium nidus* Linn.	40 6±1.89	42.2±1.2	-
3.	*Asplenium nessii* Christ	-	28.4±3 3	14.2±0 72
4.	*Arthromeris himalayansis* (Hook) Ching	-	31.3±2.9	67 5±7 1
5.	*Arthromeris wallichiana* (Spreng.) Ching	74.75±9.4	52 6±1.5	37.1±1.9
6.	*Araiostagia pulchra* (D.Don) Copel	-	41.5±1.3	-
7	*Araiostegia beddomei* (Hope) Ching	-	15 0±8 9	-
8.	*Ctenopteris subfalcata* Blume *ex* Kunze	-	-	22.5±0.9
9.	*Crypsinus grifithiana* (Hook.) Copel.	-	40.9±2.7	-
10.	*Drynaria quercifolia* (L.) J.Sm.	43.3±2.3	12.2±0.7	-
11.	*Hymenophyllum simonsianum* Hook.	-	-	14.2±1.7
12.	*Lycopodium phlegmaria* Linn.	-	20.3±1.2	-
13.	*Loxogramme involuta* (D.Don) Presl	28 .3±1 21	11.6±0.5	-
14.	*Loxogramme carinata* Price	-	11.1±0.7	-
15.	*Lepisorus clathratus* (Clarke) Ching	-	17.3±1.1	-
16.	*Lepisorus mehrae* Fras.-Jenk.	-	19.2±2 1	28.5±2 0
17.	*Lepisorus loriformis* (Wall. *ex* Mett.) Ching	-	20.0±1.9	38.7±2 9
18.	*Lepisorus loriformis* var. *steniste sensu* Bedd.	-	-	33.3±1 8
19.	*Lepisorus morrisonensis* (Hayata) H. Ito	12.5±6.7	22.7±1.2	-
20.	*Lepisorus nudus* (Hook.) Ching	50.2±4.0	38.0±3.3	13.3±1 4
21.	*Lepisorus thunbergianus* (Kaulf.) Ching	40.0±2.0	30.2±1 9	9.5±0.6
22.	*Lepisorus ussuriensis* (Regel *et* Maack) Ching	-	14.2±0.3	21.3±1.7
23.	*Lepisorus bicolour* Ching	-	12.5±1.3	-
24.	*Lepisorus kuchenensis* (Wu) Ching	-	20.3±2.0	-
25.	*Lepisorus sesquipedalis* (J.Sm) Fras.-Jenk.	-	28.5±2.1	23.7±2.3
26.	*Leucostegia immersa* Wall. *ex* Presl	-	31.4±3.0	16.1±1 2
27.	*Lepidogrammitis rostrata* (Bedd.) Ching & SK Wu in Wu	-	21.6±3 2	-
28.	*Microsorium membranaceum* (D.Don) Ching	34.7±3.3	20.8±2.0	-
29.	*Microsorium punctatum* (L.) Copel.	47.7±3.3	10.0±0.5	-

30.	*Mecodiutn exsertum* (Wall. *ex* Hook) Copel.	-	26.6±1.3	33.9±2 7
31.	*Mecodium badium* (Hook. et Grev.) Copel.	-	-	23.7±1.9
32.	*Nephrolepis cordifolia* (L) Pr	73 1±8.0	51.2±5.2	16.4±1.1
33.	*Neocheiropteris normalis* (D.Don) Taga.	-	12.5±0.9	-
34.	*Neocheiropteris zipelli* (Bl.) Bosnian	-	28.7±2.1	-
35.	*Oleandra neriformis* Cav.	-	11.1±0.6	-
36.	*Oleandra wallichii* (Hook) Pr.	12.5±1.0	55.4±2.7	42.2±2.4
37.	*Pyrrosia flocculosa* (D Don) Ching	-	25.7±1.3	-
38.	*Pyrrosia lanceolata* (L.) Farwell	64.0±6.2	24.3±2 3	-
39.	*Pyrrosia manii* (Gies) Ching	28.5±2.1	14 2±1.0	-
40	*Pyrrosia nuda* Ching	40.0±3 1	10.0±0.3	-
41.	*Pyrrosia pannosa* (Mett. *ex* Kuhn) Ching	22.2±2.1	16.5±1.7	-
42.	*Polypodiodes amoenum* (Wall. *ex* Mett.) Ching	-	32.3±2.8	41 4±3 2
43.	*Polypodiodes lachnopus* (Wall. *ex* Hook.) Ching	-	22.5±2.2	-
44	*Polypodiodes microrrhizoma* (Clarke *ex* Baker) Ching	20 0±1.3	27.0±2.8	23.75±1.7
45	*Polypodiastrum argutum* (Wall. *ex* Hook.) Ching	-	22.7±2.6	14.2±1.0
46.	*Phymatopteris ebenipes* (Hook,) Pic. Ser.	-	12.5±1.0	-
47.	*Phymatosorits cuspidatus* (D.Don) Pic. Ser.	20.0±1.7	47.5±2.4	14 2±0 5
48.	*Pseudodrynaria coronans* (Wall. *ex* Hook) Ching	40.7±4.1	22.5±0.7	-
49.	*Selaginella involvens* (Sw.) Spring	-	20.0±1.3	15.4±1.5
50	*Vittaria elongata* Sw.	11.8±1.2	11.1±1.5	-
51.	*Vittaria himalayansis* Ching	-	30.0±2.8	-
52.	*Vittaria linearifolia* Ching	-	11.1±0.7	-
53.	*Vittaria ophiopogonoides* Ching	16.6±2.1	23.7±2.0	-
54	*Vittaria taeniophyla* Copel	-	33.3±4 1	34.2±4.3

The tree top region had a single epiphytic pteridophyte. The species found in low and middle hill forest zone develop marked contrivances for storing their available water forming nest like or bracket like structures and thick cuticularised leaves [*Pseudodrynaria coronans* (Wall. ex Hook) Ching, *Drynaria quercifolia* (L.) J. Sm., [*Asplenium nidus* Linn], by inrolling of leaves to prevent desiccation [*Lepisorus nudus* (Hook) Ching] and by the development of thick woollen hair on the leaf surface for preventing excessive transpiration [*Pyrrosia pannosa* (Mett. ex Kuhn) Ching]. Succulent epiphytes like, *Microsorum punctatum* (L.) Copel, *Pyrrosia* sp., *Vittaria* sp.and *Asplenium nidus* Linn. have physiological adaptation by operating CAM (Crassulacean acid metabolism)[28-29].

The host trees found in the low and middle hill forest zone were *Shorea robusta* Gaertn.f., *Schima wallichii* Choisy, *Bauhinia purpurea* Linn. *Cedrela toona* Roxb.*ex* Rottl., *Pterospermum tetragonum* DC., *Salmalia malabarica* (DC.) Schott and Endl., *Sterculia vittosa* Roxb., *Terminalia myriocarpa* Van Heurck and Muell. Arg, *Albizia lebbeck* (L.) Benth., *Tectona grandis* Linn. The average height of the host trees was 30m. Among the 21 epiphytic pteridophytes, *Pseudodrynaria coronans* (Wall ex Hook) Ching *Microsorum punctatum* (L) Copel
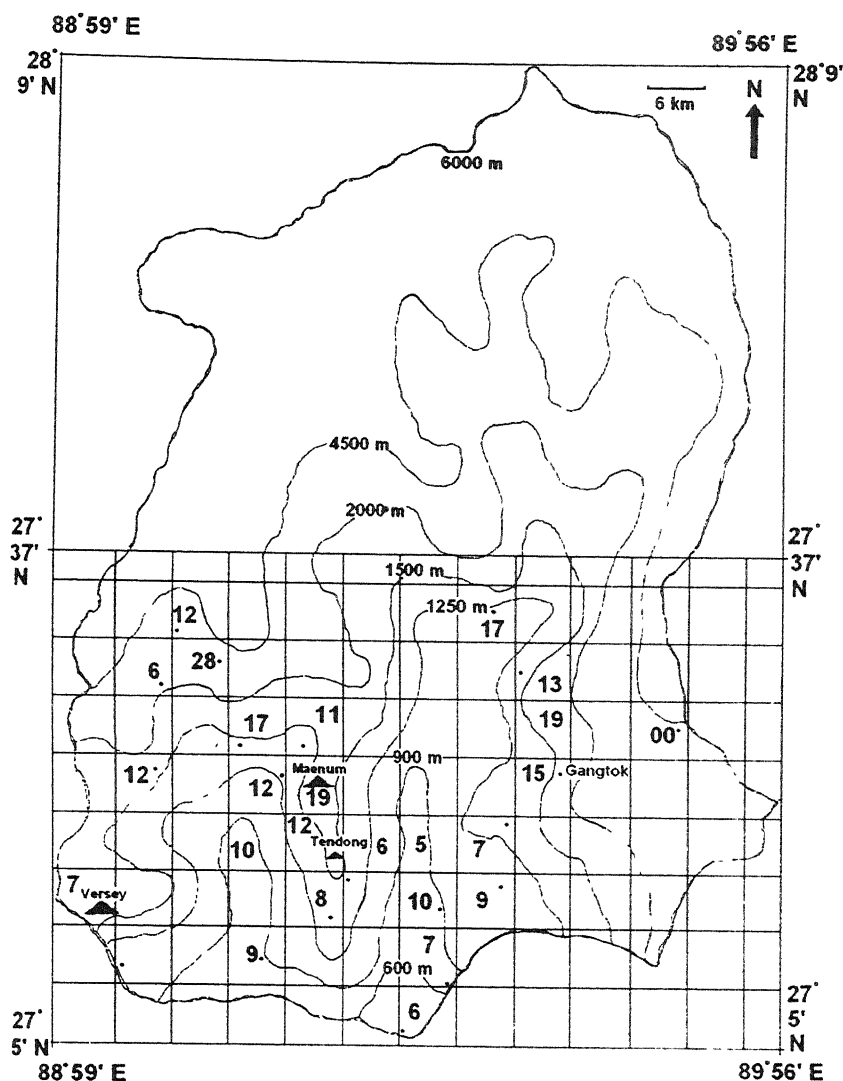
Fig. 1 - Map of Sikkim showing distribution of number of epiphytic pteridophytic species in the grid cells along different altitudes.

and *Lycopodium sguarrosum* had restricted distributions on trees, while other species were distributed in two or three vertical regions (Table 2).

Upper hill forests consisted of host trees such as *Quercus lamellosa* Sm., *Q. pachyphylla* Kunz, *Q. lineata* Blume, *Magnolia campbelli* Hook. f. and Thoms., *Betula alnoides* Buch.-Ham., *Acer calmpbelli* Hook.f. and Thoms, *Rhododendron griffIthianum* Wight, with an average height of 24 m. This region was the ideal habitat of epiphytic pteridophytes harbouring diverse types of pteridophytes. Temperature of this region ranged from 5°C-18°C, almost throughout the year, except in winter season; high humidity and heavy rainfall, which measure about 282 cm annually (average of 28 grid cells) prevailed during monsoon, provided ideal growth conditions for epiphytic pteridophytes. Fifty species of epiphytic pteridophytes were recorded from this zone (Table 2). Among these, only *Oleandra wallichii* (Hook.) Pr. was found to be distributed widely in all the four vertical regions of the host trees while, *Nephrolepis cordifolia* (L.) Pr., *Lepisorus ussuriensis* (Regel *et* Maack) Ching, *Polypodiastrum argutum* (Wall. *ex* Hook.) Ching, *Pseudodrynaria coronans* (Wall. *ex* Hook.) Ching, *Pyrrosia manii* (Gies.) Ching, *Selaginella involvens* (Sw.) Spring and *Ctenopteris subfalcata* Blume *ex* Kunze were found to have restricted vertical distributions in either the foot region or in the upper foot region. *Polypodioides lachnopus* (Wall. *ex* Hook.) Ching was found to have wide vertical distributions except in the

tree top region. *Lepisorous mehrae* and *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk. were found in tree top region in addition to *Oleandra wallichii* (Hook.) Pr.

Asplenium ensiforme Wall. *ex* Hook., *Lepisorus loriformis* (Wall. *ex* Mett.) Ching, *Lepisorus loriformis* var. *steniste* sensu Bedd., *Crypsinus griffithiana* (Hook.) CopeL, *Vittaria ophiopogonoides* Ching, *V linearifolia* Ching, *V. taeniophylla* Copel, *V. himalayansis* Ching and *V. elongata* Sw. were found to survive in the months of November and December with fertile leaves. They showed some adaptive features like thick fleshy leaves with highly cuticularised epidermal layer or narrow lanceolate leaves, which helped them to withstand the adverse conditions. Out of the total 50 species occurring in the upper hill forest zone, 45 were found in the upper foot region and 33 in the branch region of the host trees (Table: 3).

Rhododendron-Conifer zone forests of Sikkim are situated between 2700-3600 m altitudes. Temperature of this zone is almost below 10°C throughout the year, except in winter months, when it is below freezing temperature. This zone consisted of host trees such as *Quercus lineata* Blume, *Acer campbelli* Hook.f. and Thoms, *Betula utilis* D.Don, *Magnolia campbelli* Hook.f. and Thoms, *Rhododendron arboreum* Sin., *R. campanulatum* Don, *Tsuga brunoniana* Carr., *Abies densa* Griff., *Cryptomeria japonica* D.Don, *Pinus* spp., *Rhododendron setosum* D.Don. The average height of the host trees of this region was 18 m. This zone harbours only 24 epiphytic pteridophytes. Out of them, *Oleandra wallichii* (Hook.) Pr., *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk. and *Arthromeris wallichiana* (Spreng.) Ching, had wide vertical distributions, except at the tree top region. *Lepidogrammitis rostrata* (Bedd.) Ching and Sk Wu in Wu. *Ctenopteris subfalcata* Blume *ex* Kunze, *Hymenophyllum simonsianum* Hook, and *Selaginella involvens* (Sw.) Spring, were found to have very restricted vertical distributions. It was observed that in the Rhododendron-Conifer zone, *Rhododendron* spp. and some conifers did not harbour much epiphytic pteidophytes. Only two pteridophytic species [*Arthromeris himalayansis* (Hook.) Ching and *Lepisorus loriformis* (Wall. *ex* Mett.) Ching], were found to grow on trunks of *Rhododendron* spp. having smooth type of bark. *Quercus lineata* Blume common in this region acted as good host for epiphytes due to the corrosive nature of the bark.

Table 2 shows that the upper foot region of each of the three-altitudinal zones have the maximum diversity of pteridophytes. Species richness (a simple count of the number of species), was far greater in the upper foot and branch regions than the lower foot and tree top regions. In the low and middle hill forest zones tree top region showed poorest occurrence of epiphytic pteridophytes. The highest diversity of epiphytic pteridophytes was in the upper foot regions of the host trees in the upper hill forest area.

Analysis of correlation between species richness with different environmental and geographical factors showed that correlation value with rainfall ($r^2 = 0.3278$), relative humidity ($r^2 = 0.2825$), annual minimum and maximum temperatures ($r^2 = 0.0075$ to 0.1475) and altitude ($r^2 = 0.004$) varies considerably.

Discussion

Epiphytic pteridophytes are represented in southern Sikkim by 53 species and one variety. Polypodiaceae is the largest and dominant family of this area represented by 31 species and one variety, followed by Vittariaceae with five species and Aspleniaceae, Hymenophyllaceae and Davalliaceae each represented by three species. Other epiphytic families are represented by one or two species.

All the epiphytic members are distributed in the different altitudinal regions (Fig. 1). The diversity of epiphytic pteridophytes increases with the altitude up to a certain level. From 2700 m upward, diversity of epiphytic pteidophytes decreases. Actually, in low and middle hill forest zone the prevailing adverse conditions like high temperature (average 18°C-35°C throughout the year, except in winter), low humidity etc. hinder the growth of epiphytic pteidophytes and decrease the chances of epiphytic establishment and growth. Relative humidity (RH) is a vital component for the establishment of epiphytic habit. Incidence of poor epiphytic habit in low and middle hill forest zone and in coniferous forest zone results from the prevalence of deciduous type of trees and reduced leaved coniferous type of trees respectively. On the contrary, rich epiphytic habit, which is present in the upper hill forest zone, results due to the presence of evergreen broad-leaved forest trees. Statistical analysis also support this view. From statistical data analysis it was found that, epiphytic species richness was highly correlated with average annual rainfall ($r^2 = 0.3278$) and average relative humidity ($r^2 = 0.2825$). Weaker correlations were found in case of annual average temperature ($r^2 = 0.0075$ to

Table 2 - Epiphytic species distribution in different regions of the host trees in relation to the different altitudinal forest types.

Sl. No.	Epiphytic pteridophytes	Low & middle hill forest zone (upto 1500m)				Upper hill forest zone (1500-2700 m)				Rhododendron conifer zone (2700-3600)			
		R I	R II	R III	R IV	R I	R II	R III	R IV	R I	R II	R III	R IV
1.	*Asplenium ensiforme*	-	-	-	-	-	+	+	-	-	+	+	-
2.	*Asplenium nidus*	-	+	+	-	-	+	+	-	-	-	-	-
3.	*Asplenium nessi*	-	-	-	-	-	+	-	-	-	+	-	-
4.	*Arthromeris himalayansis*	-	-	-	-	-	-	-	-	-	+	-	-
5.	*Arthromeris wallichiana*	+	+	-	-	-	+	+	-	+	+	-	-
6.	*Araiostegia pulchra*	-	-	-	-	+	+	-	-	-	-	-	-
7.	*Araiostegia beddomei*	-	-	-	-	+	-	-	-	-	-	-	-
8.	*Ctenopteris subfalcata*	-	-	-	-	-	-	-	-	+	+	-	-
9.	*Crypsinus grifithiana*	-	-	-	-	-	+	+	-	-	-	-	-
10.	*Drynaria quercifolia*	-	+	+	-	-	+	+	-	-	-	-	-
11.	*Hymenophyllum simonsianum*	-	-	-	-	-	-	-	-	-	+	+	-
12.	*Lycopodium phlegmaria*	-	-	-	-	-	+	-	-	-	-	-	-
13.	*Loxogramme involuta*	-	+	+	-	-	+	+	-	-	-	-	-
14.	*Loxogramme carinata*	-	-	-	-	-	+	+	-	-	-	-	-
15.	*Lepisorus clathratus*	-	-	-	-	-	+	-	-	-	-	-	-
16.	*Lepisorus mehrae*	-	-	-	-	-	-	+	+	-	-	+	-
17.	*Lepisorus loriformis*	-	-	-	-	-	+	+	-	-	+	-	-
18.	*Lepisorus loriformis* var. *steniste*	-	-	-	-	-	-	-	-	-	+	-	-
19.	*Lepisorus morrisonensis*	-	+	-	-	-	+	-	-	-	-	-	-
20.	*Lepisorus nudus*	+	+	+	-	+	+	+	-	+	+	-	-
21.	*Lepisorus thunbergianus*	-	+	+	-	+	+	+	-	+	+	-	-
22.	*Lepisorus kuchenensis*	-	-	-	-	-	+	+	-	-	-	-	-
23.	*Lepisorus sesquipedalis*	-	-	-	-	-	+	-	-	-	+	+	-
24.	*Lepisorus ussuriensis*	-	-	-	-	-	+	-	-	-	+	+	-
25.	*Lepisorus bicolour*	-	-	-	-	-	+	-	-	-	-	-	-
26.	*Leucostegia immersa*	-	-	-	-	+	+	-	-	-	+	+	-
27.	*Lepidogrammitis rostrata*				-	+	+	-	-	-	-	-	
28.	*Microsorium membranaceum*	-	+	+	-	-	+	+	-	-	-	-	-
29.	*Microsorium punctatum*	-	-	+	-	-	+	+	-	-	-	-	-

No.	Species												
30.	*Mecodium exsertum*	-	-	-	-	+	+	-	-	+	+	-	-
31.	*Mecodium badium*	-	-	-	-	-	-	-	-	+	+	-	-
32.	*Nephrolepis cordifolia*	+	+	-	-	+	-	-	-	+	-	-	-
33.	*Neocheiropteris normalis*	-	-	-	-	-	+	+	-	-	-	-	-
34	*Neocheiropteris zipelli*	-	-	-	-	-	+	+	-	-	-	-	-
35.	*Oleandra neriformis*	-	-	-	.	-	+	-	-	-	-	-	-
36.	*Oleandra wallichii*	+	+	+	-	+	+	+	+	-	+	+	+
37	*Pyrrosia flocculosa*	-	-	-	-	-	+	+	-	-	-	-	-
38.	*Pyrrosia lanceolata*	-	+	+	-	-	+	+	-	-	-	-	-
39.	*Pyrrosia manii*	-	+	+	-	-	+	-	-	-	-	-	-
40	*Pyrrosia pannosa*	-	+	+	-	-	+	+	-	-	-	-	-
41	*Pyrrosia nuda*	-	-	-	-	-	+	+	-	-	+	-	-
42.	*Polypodiodes amoenum*	-	-	-	-	-	+	+	-	-	+	-	-
43.	*Polypodiodes lachnopus*	-	+	+	-	-	+	+	-	-	+	+	-
44.	*Polypodiastrum argutum*	-	-	-	-	-	+	+	-	-	-	-	-
45.	*P microrrhizoma*	-	-	-	-	-	+	+	-	-	+	+	-
46.	*Phymatopteris ebenipes*				-	+	+	-	-	-	-	-	
47.	*Phymatosorus cuspidatus*	-	+	-	-	-	+	-	-	-	+	-	-
48	*Pseudodrynaria coronans*	-	+	-	-	-	+	+	-	-	-	-	-
49.	*Selaginella involvew*		-	-	-	+	-	-	-	+	-	-	-
50.	*Vittaria elongata*	-	+	+	-	-	+	+	-	-	-	-	-
51.	*Vittaria himalayansis*	-			-	+	+	-	-	-	-	-	
52.	*Vittaria linearifolia*	-	-	-	-	-	+	+	-	-	-	-	-
53.	*Vittaria ophiopogonoides*	-	+	+	-	-	+	+	-	-	-	-	-
54.	*Vittaria taeniophyla*	-	-	-	-	-	+	+	-	-	+	+	-

Table 3 - Epiphytic species-richness at different regions of the host plant in relation to altitudinal forest types.

Forest types along the altitudes	Height section of the host tree				Total epiphytic species
	Foot region (I)	Upper foot region (II)	Branch region (III)	Tree top region (IV)	
Low and middle hill forest (upto 1 500 m) zone	04	18	14	00	20
Upper hill forest zone (1500-2700m)	10	45	33	02	50
Rhododendron-conifer forest zone (2700-3600m)	09	23	10	01	24

NB: The same epiphyte may develop in more than one region.
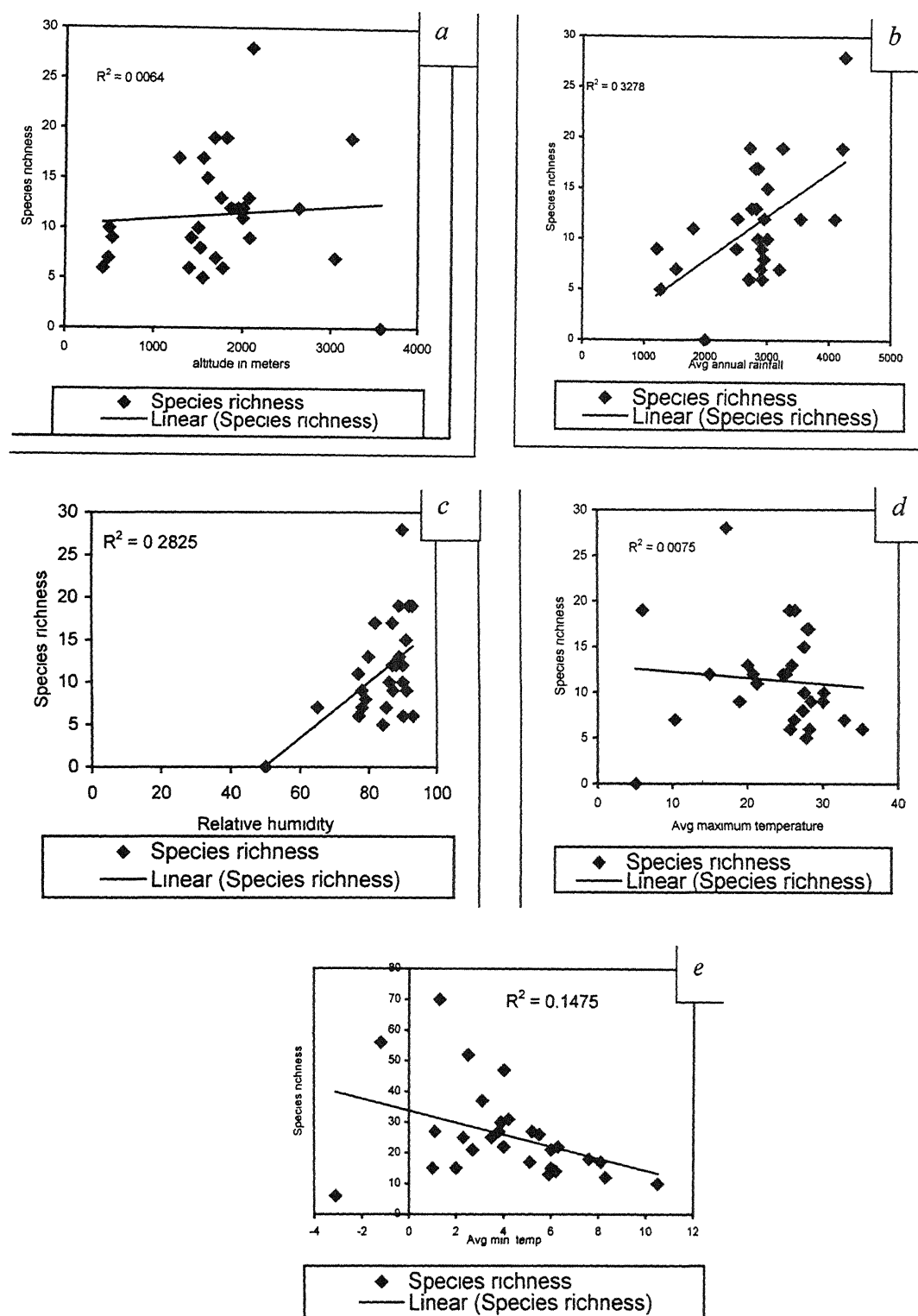
Fig. 2 - a. Variation of epiphytic species richness along the altitudinal gradient in South Sikkim. b. Change of epiphytic species richness with average annual rainfall in South Sikkim. c. Change of epiphytic species richness with relative humidity. d. Effect of average maximum temperature on epiphytic species richness of different places of South Sikkim. e. Effect of average minimum temperature on epiphytic species richness of different places of South Sikkim.

r^2 = 0.1475) and altitudinal variations (r^2 0.004). Only a few species like *Pseudodrynaria coronans* (Wall, *ex* Hook.) Ching, *Drynaria quercifolia* (L.) J.Sm., *Lepisorous nudus* (Hook.) Ching and *Pyrrosia pannosa* (Mett. *ex* Kuhn.) Ching and some other species can withstand these conditions by developing the following adaptations.

i. Nest-like or bracket-like structure.

ii. Thick cuticularised leaves.

iii. Presence of thick woolen hair *etc.*

Upper hill forest zone shows the maximum diversity of epiphytic pteridophytes. About 91% of the total number of epiphytic pteridophytes is found in this zone. This zone provides the most congenial conditions of temperature (5°C-18°C) and humidity for growth of epiphytic pteridophytes. Rhododendron-Conifer zone occupies the intermediate position between Low and Middle hill and Upper hill forest zones regarding epiphytic pteridophyte diversity.

From the point of view of preference of altitudinal zones for frequency of occurrences, *Arthromeris wallichiana* (Spreng.) Ching, *Lepisorus nudus* (Hook.) Ching, *L. thunbergianus* (Kaulf.) Ching and *Nephrolepis cordifolia* (L.) Pr. show decreasing tendency in the number with the increase of altitude, whereas in *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk., it is reverse. Some species like *Oleandra wallichii, Plypodioides lachnopus* and *Phymatosorus cuspidatus* (D.Don) Pic. Ser. had maximum frequency in the mid altitude (1500-2700 m). On the other hand, species like *Ctenopteris subfalcata* Blume *ex* Kunze, *Hymenophyttum simonsianum* Hook., *Lepisorus loriformis* var. *steniste* sensu Bedd., *Mecodium badium* (Hook. *et* Grev.) Copel, are restricted to higher altitudes (2700-3600 m) with low frequency of occurrence. Sixteen species of epiphytic pteridophytes prefer only the upper hill (1500-2700 m) forest zone; they are neither found in low and middle hill forest zones, nor in the Rhododendron-Conifer zone (Table 2).

The vertical distribution of epiphytic pteidophytes is mostly confined to one or two regions on the host trees for the majority of species, except a few species, which are widely distributed throughout the three vertical regions. Depending on their wide or restricted distributions, epiphytic pteridophytes are divided into two categories: - i. Generalist species (having wide vertical distribution) and ii. Specialist species (having restricted vertical distribution). Regarding richness of epiphytic pteridophytic species on different vertical regions, upper foot region bears the maximum number of epiphytic pteridophytes. Upper foot region starts from 6 m and ranges up to 20 m. So, this region provides suitable microhabitat for growth of epiphytic pteridophytes due to the presence of high humidity, ideal range of temperatures (5°C-18°C), low wind velocity and being out of reach of grazing animals. On the other hand, the tree top region showing the lowest diversity of epiphytic pteridophytes is probably due to adverse climatic conditions like low humidity, low temperature and high wind velocity.

Epiphytic species richness is highest in the Upper hill forest zone and prevalence of epiphytes is maximum in the upper foot region of the host trees (Table 3).

As the upper hill forest areas of Sikkim harbour the highest epiphytic pteridophyte diversity this region may be considered as most suitable for the recommendation of *in situ* conservation for preserving the epiphytic pteridophyte biodiversity.

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In-situ and *ex-situ* evaluation of seaweed liquid fertilizers on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn.

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Abstract

The effects of seaweed liquid fertilizers (SLFs) prepared from *Sargassum tenerrimum* J. Ag , *Hypnea valentiae* Turn and *Solieria robusta* (Grev) Kylin on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn. were evaluated *in-situ* and *ex-situ*. Among different seaweed concentrations tested *in-situ* viz 10%, 30%, 50%, 70% and 100%, 10% extract of all the seaweeds studied was found to promote germination, hypocotyl and root elongation SLFs prepared from dried seaweeds were more effective than fresh seaweeds at all the concentrations tested. Under *ex-situ* conditions 10% *Hypnea valentiae* extract showed one to two fold increase in root length, shoot length and leaf area and two fold increase in total chlorophyll content.

Key words : seaweed extract, seed germination, hypocotyl elongation, root elongation.

सारांश

विभिन्न समुद्री शैवालो *सरगेसम टेनेरीमम* जे०एजी०, *हिप्निया व्हेलेन्शी* टर्न० एव *सोलेरिया रोबस्टा* (ग्रीव) काईलिन, से बनाए गये द्रव्य खाद (एस०एल०एफ०) का प्रभाव *ट्रायगोनेला फोईनम ग्रासियम* लिन के बीज अकुरण, आरभिक वृद्धि एव हरित द्रव्य मात्रा को अन्त अवस्थिति एव बाह्य अवस्थिति मे मूल्याकित किया गया। सभी समुद्री शैवालो के विभिन्न सान्द्रता के अर्क (जैसे कि 10%, 30%, 50%, 70% एव 100%) मे से 10% सान्द्रता का अर्क बीज अकुरण वृद्धि कारक एव हायपोकोटाईल एव मूल की लबाई का वर्धक सिद्ध हुआ। सभी सान्द्रताओ मे, सुखाये गये शैवालो का अर्क, ताजे शैवालो के अर्क से बेहतर पाया गया। बाह्य अवस्थिति मे 10% *हिप्निया व्हेलन्शी* के अर्क के कारण मूल एव तने की लबाई एव पत्ते का क्षेत्रफल एक से दो गुना हो गया तथा हरित द्रव्य की मात्रा भी दो गुनी हो गयी।

साकेतिक शब्द : समुद्री शैवालीय अर्क, बीजाकुरण, हाइपोकोटाइल का लम्बा होना, मूल का लम्बा होना।

Introduction

India, with more than 8000 km coastal stretch, harbors about 900 different seaweeds with plentiful biomass. These have hardly had been utilized by industries except for hydrocolloid production. Recently, a few seaweed utilizing industries in southern India are turning towards the large scale production of Seaweed Liquid Fertilizers (SLFs)[1]. While, historical records show that the use of seaweeds in agriculture is a very old and routine practice widely followed especially in Europe[2], its use has yet to be established in Indian agriculture. According to Thivy[3], in India there is a causal use of seaweeds for agricultural purpose in places like Ratnagiri, Goa, Karwar and Tirunellveli. In India, '*ethanophycological*' use of seaweeds as manure has also been documented very recently for coconut, mango, cashew-nut and jackfruit orchards of Malvan[4-5]. Apart from agricultural crop plants, seaweed extracts have been tested positive for vegetative growth in ornamental foliage and horticultural plants and enhancing growth of prokaryotic organisms[6-9]. The growth promoting activity of seaweed extracts is mainly due to presence of plant hormone like substances[10-13], high levels of potassium[14], vitamins[15] and micro[16-17] and macro[17-19] nutrients. Such substances become readily available to crop plants when applied directly to the soil or used as foliar sprays. Seaweed liquid fertilizers also help plants in withstanding different stress conditions and infec-

tions [20-23]. The present study was aimed at evaluating the effects of seaweed liquid fertilizers (SLFs) prepared from *Sargassum tenerrimum* J. Ag., *Hypnea valentiae* Turn. and *Solieria robusta* (Grev.) Kylin on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn. under *in-situ* and *ex-situ* conditions.

Material and Methods

The seaweeds were collected from Malvan coast (73.27 E and 16.03 N) in November, 1998. Selection of test seaweeds was made after interviewing farmers of Malvan, who have been using them traditionally, and detailed enquiry showed these as the preferred choice amongst the other available seaweeds. The seaweeds were hand picked and washed thoroughly with seawater in the field itself to remove all the unwanted impurities like epiphytes and adhering sand particles and then brought immediately to the working laboratory and washed with tap water. Half the portion from the collection was directly used for extraction of liquid fertilizer while half was shade dried before extraction. Separate fertilizer extracts were prepared from dry and fresh seaweeds following the method described by Bhosle *et al*[24]. 1000 gm of each fresh and dried algae were cut in to small pieces homogenized in 1000 ml distilled water in an electric blender and filtered. The resultant pull out is considered as the full strength extract (100%) and used in the further experiments. Different concen-
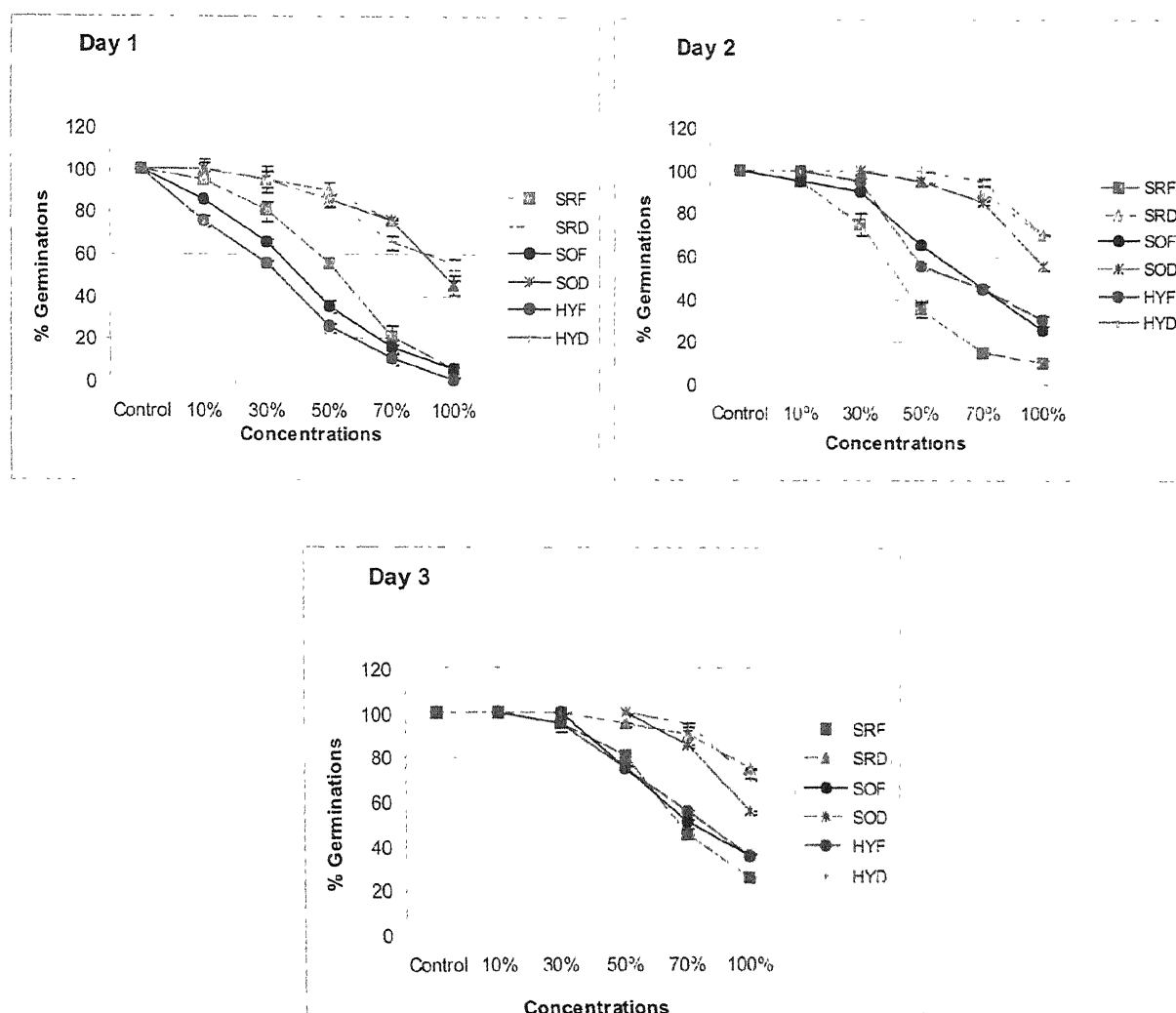
Fig. 1 - Effect of different seaweeds extracts on percentage seed germination.

*SRF : *Sargassum* Fresh extract; SRD: *Sargassum* Dry extract; SOF: *Solieria* Fresh extract; SOD: *Solieria* Dry extract, HYF: *Hypnea* Fresh extract & HYD: *Hypnea* Dry extract.

Table 1 - Effect of different seaweed extracts on hypocotyle and root elongation of *Trigonella foenum graecum*

Seaweed	Sargassum				Solieria				Hypnea			
	Hypocotyle elongation		Root elongation		Hypocotyle elongation		Root elongation		Hypocotyle elongation		Root elongation	
Concentrations	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Control	2 47	3.90	1.44	3.22	2.47	3.90	1.44	3.22	2.47	3.90	1.44	3.22
	(± 0.25)	(± 0.11)	(± 0.01)	(± 0.12)	(± 0.25)	(± 0.72)	(± 0.25)	(± 0.15)	(± 0.16)	(± 0.33)	(± 0.40)	(±0.24)
10 %	0.57	5.02	0.50	5.42	1.80	6.33	1.46	7.48	2.20	5.02	2.20	5.42
	(± 0.17)	(± 0 71)	(± 0.05)	(± 0 20)	(± 0.08)	(± 0 45)	(± 0 70)	(± 1.12)	(± 0.05)	(± 0.12)	(± 0 63)	(± 0 19)
30 %	2 00	4.65	2.42	5.95	2.68	5.66	2 24	6.63	0	4.65	2.10	5.95
	(± 0.01)	(± 0.25)	(± 0.18)	(± 1.05)	(± 0 89)	(± 0.03)	(± 0.86)	(± 0.01)	(± 0)	(± 1 20)	(± 0 05)	(±2 07)
50 %	2.48	5 36	2.93	5.75	2.55	2.48	2.04	3.56	0	5.36	2.00	5.75
	(± 0.18)	(± 1.72)	(± 0.43)	(± 1.89)	(± 1.10)	(± 0.43)	(± 0.07)	(± 1.56)	(± 0)	(± 2.14)	(± 1.67)	(±1.92)
70 %	2.88	4.89	2 96	5.24	1.15	0	1.06	2.75	0	4.89	1.80	5.24
	(± 1.64)	(± 1.93)	(± 1.20)	(± 2.04)	(± 1.02)	(± 0)	(± 1.08)	(± 1.08)	(± 0)	(± 1.46)	(± 1.21)	(±2.00)
100 %	1.39	4.99	2.04	4.69	0	0	1.30	0.35	0	4.99	0	4.69
	(± 1.12)	(± 1.28)	(± 1.07)	(± 1.87)	(± 0)	(± 0)	(± 1.01)	(± 1.07)	(± 0)	(± 2.07)	(± 0)	(±1.85)

Table 2 - Effect of 10 % dry Hypnea extract on *Trigonella foenum graecum* on growth parameters

Parameters	SLF Treatment	
	Control	Treated
Fresh wt (g)	0.25 (± 1.01)	0.87 (± 1.27)
Dry wt (g)	0.04 (± 0.01)	0.13 (± 0.08)
Shot length (cm)	11.79 (± 4.21)	13.63 (± 5.89)
Root length (cm)	5.1 (± 1.47)	8.02 (± 2.06)
Leaf area (mm^2)	532 (± 68.92)	769 (± 84.06)
Chlorophyll *a* (mg/g Fresh wt)	0.10 (± 0.04)	0.09 (± 0)
Chlorophyll b (mg/g Fresh wt)	0.06 (± 0)	0.12 (± 0.03)
Total Chlorophyll (mg/g Fresh wt)	0.16 (± 0.02)	0.22 (± 0.05)

trations of the seaweed extracts viz. 10%, 30%, 50%, 70% and 100% were made using distilled water. All the tests were carried out in duplicate.

Healthy, uniform seeds of *Trigonella foenum graecum* were pre-soaked in distilled water (control) and extracts of respective concentrations for 24 h and then equispacially placed (n = 10) in glass Petri dishes of 4" diameter with Whatman filter paper No. 1 at the base. Ten ml of different concentrations of the SLFs was then poured in the Petri dishes. Distilled water was taken as control. Seed germination % was recorded for three successive days. Root and hypocotyl elongation were measured after eight days. Lower (10%) concentration of dry *H. valentiae* extract, which showed encouraging results, was selected for further *ex-situ* studies in the field. *Trigonella foenum graecum* plants were grown in two different plots side by side in the

Botanical garden, Department of Botany, University of Pune. Plants were watered every day for 30 days. In one plot, plants were applied 10% of dry *H. valentiae* extract as foliar drench on alternate days. The other plot was maintained without any foliar drench (control). At the end of the experiment, when plants were at their vegetative growth stage, five random plants from each plot were sampled and study was made of fresh weight, dry weight, shoot length, root length, leaf area and chlorophyll.

Results and Discussion

In-situ evaluation of seed germination against varying concentrations of the seaweed extracts (Figure 1) showed 100 % seed germination in control on first day itself. Seeds treated with algal extracts showed maximum germination % only at lower concentrations. In case of dry extract of *Sargassum tenerrimum, Hypnea valentiae* and *Solieria robusta* 100 % seed germination was achieved in 10% concentration on the first day. The seeds treated with 10 % fresh extract of *Sargassum tenerrimum, Hypnea valentiae* and *Solieria robusta* showed 95 %, 85 % and 75 % germination respectively. Longer exposure to SLFs increased seed germination. Minimum % seed germination was recorded for fresh extract of *Sargassum tenerrimum* and maximum for dry extract of *Hypnea valentiae*. Similar results were also recorded for maize[25], green chillies and turnip[26], okra and tomato[27]. When treated with 10 % extract of *Caulerpa racemosa* and *Gracilaria edulis, Vigna catajung* has been reported to show 100 % seed germination[28]. Williams *et al.*[29] have reported gibberellin in seaweed extracts. At low concentrations gibberellin acts on seed tissue leading to increase in cell division and cell elongation, which enhance seed germination. Similar effects of gibberellin have been reported in cereals[30].

Root as well as hypocotyl elongation was found to be more in dry extracts that in fresh extracts made of *Sargassum tenerrimum, Hypnea valentiae* and *Solieria robusta* (Table 1). These observations are in agreement with earlier studies[24, 31]. In 100 % fresh as well as dry and 70 % dry *Solieria robusta* extract and 30 %, 50 %, 70 % and 100 % *Hypnea valentiae* extract showed no hypocotyl elongation. However, in 100 % fresh *Hypnea valentiae* extract no root initiation was observed. The hypocotyl elongation could be the result of certain sugars present in the extract as suggested by Blunden and Woods[32]. These sugars may serve as energy source during early developmental stages of the seedling. However, presence of biological molecules like Phenylaceticacid (PAA) in the extracts might promote the hypocotyl elongation at lower concentrations[31]. Auxins, cytokinins, cis and trans ribosylzeatin, transzeatin, dihydrozeatin and N super(6) (Delta super(2)-isopentenyl)adenosine have been recorded in the seaweed extracts[33,34]. The root as well as hypocotyl elongation has been suggested to be controlled by these hormones at lower concentrations[35,36]. At the same time, higher concentrations of these molecules might act adversely[37].

Ex-situ evaluation of 10 % dry *Hypnea valentiae* extract showed three-fold increase in fresh as well as dry weight, while one- to two-fold increase in root length, shoot length and leaf area, and two-fold increase in total chlorophyll content of *Trigonella foenum graecum* plants. At lower concentrations, two- to three-fold increase in fresh weight of shoots and roots as well as leaf area was observed in *Cyamposis tetragonoloba* when treated with dry *Caulerpa scalpelliformis* and *Gracilaria corticata* extracts[38]. Similar observations have been made for cereals and millets[39].

Effortless decomposition of seaweeds provide certain kinds of essential minerals and amino acids[40,41]. Presence of oligosaccharides in seaweed extracts and nitrate availability of organic nitrogen along with other factors such as trace metals, phyto-hormones may cumulatively augment biomass increase[42]. Enhancement in leaf pigments in plants treated with seaweed extract has also been recorded by previous workers[23,38,39,43].

Seaweeds, which are very abundant along Indian coast, could be best utilized for our agriculture. Increasing number of seaweed polysaccharide producing industries are opting for production of seaweed liquid fertilizers[1]. Large-scale production of liquid fertilizers from this under-utilized marine renewable resource would not only help in gaining improved crop production but also provide employment to the local population.

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Natural incidence of fusarial mycotoxins

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Abstract

An extensive and intensive survey of foods (maize and sorghum) of different regions of AP was carried out for the presence of different fusarial species and their toxins. In general all the foods analysed contained one or other species of *Fusarium* The degree of mycotoxin contamination varied with the commodity and place of collection. Fumonisins, zearalenone, T_2 toxin, DON and DAS were some of the toxins detected

Key words : fusarial toxins, zearalenone, T_2-toxin, fumonisins, *Fusarium* spp

सारांश

आध्र प्रदेश के विभिन्न क्षेत्रो मे मक्का तथा ज्वार जैसे भोज्य पदार्थो पर विभिन्न फ्यूसेरियल प्रजातियो तथा इनके द्वारा उत्पन्न विषैले पदार्थो की उपस्थिति का व्यापक तथा सघन सर्वेक्षण किया गया। सामान्य रूप में सभी भोज्य पदार्थों के विश्लेषण पर, कोई न कोई *फ्यूसेरियम* की प्रजाति मिली है। माइकोटाक्सिन के सदूषण की डिग्री भोज्य पदार्थ तथा उसके सग्रह के स्थान के अनुरूप ही विभिन्नता दर्शाती रही है। जिन प्रमुख विषैले पदार्थो को प्राप्त किया गया उनमे से कुछ है, फ्यूमोनिसिन, जियरालिनोन, टी-2 विष, डान और डास।

सांकेतिक शब्द : फ्यूसेरियल विष, जेरालीनोन, टी-2 विष, फ्यूमोनिसिन, *फ्यूसेरियम* स्पीसीज

Introduction

Mycotoxin contamination of foodstuffs is posing a great threat to public health due to its potent toxic nature and fairly widespread occurrence. Hardly any edible substance can be regarded as absolutely safe from mycotoxin contamination. As a matter of fact nature of toxigenic strain coupled with environmental conditions determine the incidence of mycotoxins in food commodities. During last decade several reviews have appeared on the mycotoxin contamination of food commodities from different parts of the world[1-3]. Natural occurrence of fusarial toxins has been reported from different parts of the world[4-10]. However, such reports from this region are lacking. Therefore, an intensive and extensive survey of foods (sorghum and maize) both in the field and in storage, for fusarial infestation and contamination with their toxic metabolites were analysed.

Material and Methods

Maize and sorghum seed samples from three geographical regions of Andhra Pradesh were collected in sterilized polythene bags and brought to the laboratory. Condition of the samples and their place of collection were recorded carefully. Ten gm of grain sample was incubated at saturated atmosphere to induce mould growth for 7 days. At the end of incubation period the sample was mildly heated and extracted with water : acetonitrile (100 : 50) for detection of different mycotoxins. Methods of Kamimura *et al.*[11] and Rao *et al*[12] were adopted for detection of different fusarial toxins.

The filtrate was extracted twice with 100 ml of chloroform. The combined extracts were then passed through anhydrous Na_2SO_4 bed to remove moisture and evaporated to dryness and redisolved in 1 ml of chloroform and spotted on TLC plates and developed in different solvent systems. Subsequently, they were dried at room temperature for 5-10 minutes and observed under long wave UV light (365 nm). The toxins were confirmed chemically by spraying the plates with different spray reagents as suggested by Kamimura *et al.*[11] and others[13-16]. Different toxins were identified with the help of colour of spot and Rf value as compared with standard data (Table 1).

Results and Discussion

Out of 141 samples of maize and sorghum collected from different regions of A.P., and analysed to

Table 1 - Detection of trichothecenes and other mycotoxins produced by *Fusarium*

Name of the toxin	Solvent system	Spray reagent	Detection	
			UV	Visible
Deoxynivalenol (DON)	C : M (97 : 3)	4,7,8	-, ch, bl	y,-,-
Diacetoxy scirpenol (DAS)	C . M (97 : 3)	6, 9	bg,-	-, br
Fusarenone - X	C : M (97 : 3)	8	bl	-
HT-2 toxin	C . M (97 : 3)	6	bg	-
Moniliformin	C : M (97 : 3)	2, 10	-	br, br
Nivalenol (NIV)	C : M (97 : 3)	4, 7, 8	-, chl, bl	y,-, -
T-2 Toxin	C : M (97 · 3)	6,9	bg,-	-,P
Zearalenone	C . M (97 : 3)	1, 2, 3, 5, 7, 8	-, -, -, br, ch, bl	br, do, ip, -, -, -
Fumonisins (FBI, FB2)	W : M (3 : 1)	4,11		br

Solvents : C = Chloroform; M = Methanol, W - Water

Spray reagents : 1 = $Ce(SO_4)2$ 1% in $6N.H_2SO_4$; 2 = 2,4-DNP; 3 = $FeCl_3$ 3% in ethanol; 4 - p-anisaldehyde; 5 - 50% H_2SO_4 in methanol; 6 = 20% H_2SO_4; 7 = H_2SO_4; 8 = 20% $AlCl_3$; 9 = Chromatropic acid; 10 = 0.1% methanolic ninhydrdin; 11 = Ninhydrin

Detection colours · bl = Blue; ch = Charring, y = Yellow; bg = Blue green, br = Brown; p = Purple; do = Dark orange; lp = light purple; pi = Pink; bb = Bright blue.

get 52 samples contaminated with one or other fusarial toxins. Some of the samples were contaminated with more than one mycotoxin. Out of 8 samples of sorghum collected from Khammam, 4 were infested by either *F. moniliforme* Sheldon, *F. oxysporum* Schlecht or *F. equisetti* Corda. One sample was contaminated by fumonisins Bl, ZEA and DAS, while others were contaminated with ZEA, T-2 and DON. The samples infested with *F. equiseti* Corda. were found to contain ZEA and DON. Out of 6 samples of maize collected from Khammam were infested by 3 species of *Fusarium* and 2 samples were found to be contaminated with their respective toxins. One sample was contaminated with fumonisins, ZEA and T-2 toxin, while other samples were infested by both *F. oxysporum* Schlecht and *F. solani* Mourt. In this sample ZEA, T-2, DON and DAS could be detected. Out of 12 and 8 samples, 5 and 3 of sorghum and maize, respectively were infested by *F. moniliforme* Sheldon, *F. oxysporum* Schlecht, *F. acuminatum* Ellis and Everth and *F. equiseti* Corda. One sample was contaminated by fumonisins, ZEA, DON and DAS, while other samples were contaminated with DON only. Rest of the samples were contaminated with ZEA, T-2, DON and DAS. Out of 7 samples of sorghum collected from Karimnagar 3 samples were infested by *F moniliforme* Sheldon and *F. oxysporum* Schlecht. One sample was contaminated by ZEA and DON, the other two samples were contaminated by fumonisins B_1, B_2 and T-2 toxin. Out of 5 samples of maize, one sample was infested by *F. equiseti* Corda but none of the mycotoxins could be detected. One sample out of 5 samples of sorghum collected from Nalgonda was infested by *F. moniliforme* Sheldon and *F. heterosporum* Nees and contained fumonisins B_1 and ZEA. Two samples of maize, out of 5 collected from Adilabad, were contaminated by fumonisins B_1, zearalenone T-2 toxin DON, DAS and supported the growth of *F. moniliforme* Sheldon. Out of 6 samples of sorghum collected from Adilabad, only two samples were contaminated either by zearalenone or DON, while out of 8 samples of maize, 3 were contaminated by fumonisins, zearalenone, T-2 toxin and DAS. Two samples of sorghum out of 7 samples, collected from Nizamabad were contaminated by fumonisins, zearalenone and T-2 toxin. Similarly 2 out of 6 samples of maize were contaminated by zearalenone, T-2 toxin and DON. None of the 3

Table 2 - Natural incidence of fusarial mvcotoxins in sorghum and maize kernels.

Place of collection	Name of the commodity	No. of samples screened	No. of positive samples	Name of the fungus	Name of the toxin					
					FB1	FB2	ZEA	T-2	DON	DAS
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
Adilabad	Sorghum	6	2	*F. moniliforme*	--	--	+	--	+	--
				F. oxysporum	--	--	+	--	+	--
				F. equiseti	--	--	+	+	--	--
	Maize	8	3	*F. moniliforme*	+	--	+	--	--	+
				F. oxysporum	--	--	+	+	--	--
				F. semitectum	--	--	--	+	--	+
Cuddapah	Sorghum	2	1	*F moniliforme*	--	--	--	--	--	--
	Maize	4	3	*F. moniliforme*	+	--	--	--	--	--
				F. solani	--	--	+	--	--	--
				F. semitectum	--	--	+	+	--	--
E. Godavari	Sorghum	8	3	*F. moniliforme*	+	+	--	--	--	--
				F solani	--	--	+	+	--	+
	Maize	12	5	*F moniliforme*	--	--	+	--	--	--
				F acuminatum	--	--	--	--	--	+
Karimnagar	Sorghum	7	3	*F. moniliforme*	+	+	--	+	--	--
				F oxysporum	--	--	+	--	+	--
	Maize	5	1	*F equiseti*	--	--	--	--	--	--
Khammam	Sorghum	8	4	*F. moniliforme*	+	--	+	--	--	+
				F. oxysporum	--	--	+	+	+	--
				F equiseti	--	--	+	--	+	+
	Maize	6	2	*F moniliforme*	+	--	+	+	--	--
				F. solani	--	--	+	--	+	--
				F oxysporum	--	--	+	+	--	+
				F. semitectum	--	--	+	+	+	--
Nalgonda	Sorghum	5	1	*F. moniliforme*	+	--	+	--	--	--
				F. heterosporum	--	--	+	+	--	+
	Maize	5	2	*F. moniliforme*	+	--	+	+	+	+
				F. heterosporum	--	--	+	--	--	--
Nizamabad	Sorghum	7	2	*F moniliforme*	+	--	+	--	--	--
				F. oxysporum	--	--	--	+	--	--

[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
				F equiseti	--	--	+	+	--	--
	Maize	6	2	*F moniliforme*	+	--	--	+	--	--
				F oxysporum	--	--	+	--	+	--
				F. semitectum	--	--	+	+	--	--
Prakasham	Sorghum	3	2	*F moniliforme*	+	--	+	--	--	--
				F oxysporum	--	--	--	+	--	+
				F acuminatum	--	--	+	+	--	--
	Maize	5	2	*F moniliforme*	+	--	--	+	+	--
				F. oxysporum	--	--	--	--	+	--
				F semitectum	--	--	+	--	--	--
Rangareddy	Sorghum	3	1	*F. moniliforme*	--	--	--	--	--	--
	Maize	4	1	*F moniliforme*	+	+	+	--	--	--
				F oxysporum	--	--	--	+	--	--
				F solani	--	--	+	--	--	+
Warangal	Sorghum	12	5	*F moniliforme*	+	--	+	--	+	+
				F oxysporum	--	--	+	+	--	+
				F acuminatum	--	--	--	--	+	--
	Maize	8	3	*F. moniliforme*	+	+	+	--	--	+
				F oxysporum	--	--	+	--	+	+
				F equiseti	--	--	+	--	+	--
W. Godavari	Sorghum	5	2	*F. moniliforme*	--	+	--	--	--	--
				F. semitectum	--	--	--	--	--	--
	Maize	9	4	*F. moniliforme*	+	--	--	--	--	--
				F oxysporum	--	--	+	+	--	--
				F solani	--	--	+	--	--	--

samples of sorghum collected from Ranga Reddy were found to be show fusarial toxin contamination but they were infested with *F. moniliforme* Sheldon. Out of 4 samples of maize one sample was found to contain fumonisins, zearalenone, T-2 toxin and DAS and supported the growth of *F. moniliforme* Sheldon. Similarly one out of 2 samples of sorghum collected from Cuddapah was found to be infested by *F. moniliforme* Sheldon but contained no mycotoxin, while out of 4 samples of maize 3 were infested by *F moniliforme* Sheldon, *F. solani* Mourt and *F. semitectum* Berk and Ravenel and contained zearalenone and T-2 toxin. *F. moniliforme* Sheldon was found to produce fumonisins B, and B2 while other samples contained zearalenone and T-2 toxin. Two out of 3 samples of sorghum and 4 and 5 maize samples collected from Prakasham were infested by *F. moniliforme* Sheldon, *F. oxysporum* Schlecht and *F. acuminatum* Ellis and Everth and produced fumonisins, zearalenone, DON and DAS. Out of 8 samples of sorghum collected from East-godavari, 3 samples were infested by either *F. moniliforme* Sheldon or *F. solani* Mourt and contained fumonisins, T-2 and DAS. Five out of 12 samples of maize were contaminated by either zearalenone or DAS

and infested by either *F. moniliforme* Sheldon or *F. acuminatum* Ellis and Everth. Out of 5 samples of sorghum collected from West-godavari, 2 were contaminated by fumonisins B_2 only and infested by *F. moniliforme* Sheldon and *F. semitectum* Berk and Ravenel. Out of 9 samples of maize 4 were contaminated by one or other mycotoxins and infested by *F. moniliforme* Sheldon, *F oxysporum* Schlecht or *F. solani* Mourt.

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Promotion and cultivation of *Thysanolaena maxima* (Roxb.) Kuntze : A multiuse species in Uttarakhand

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Abstract

The natural resources form major components of life-support system in hilly areas. They include forests, livestock, non-timber forest produce, agricultural and grazing lands etc Livestock plays a major role in sustaining the life-support system in the region that largely operates at subsistence levels The rural community - particularly women in Uttarakhand, bear heavy burden as they are entrusted with various indoor and outdoor tasks, such as collection of water, fuelwood and fodder from long distances. The availability of fodder has qualitative, quantitative and technological constraints. Domestication of *Thysanolaena maxima* (Roxb.) Kuntze (broom grass), a multiuse species of high fodder value, by the rural communities is suggested as a solution. Nurseries of this grass were established at Nainital and different villages within the district to promote it as a nutritious fodder and to show its potential value as 'biological screen', 'potted house plant', and for production of 'brooms' for household use, and also as a plant of medicinal use. The adoption of this economically important grass can reduce the drudgery of rural women of Uttarakhand by strengthening their financial condition. For its popularisation amongst villagers, a number of workshops, and awareness programmes were conducted and publicity material was also produced and distributed to the rural folk of this region.

Key words: broom grass, central Himalaya, fodder, cottage industry

सारांश

पहाडी क्षेत्रो मे जीवन निर्वाह विधियो के मुख्य तत्वो मे प्राकृतिक ससाधनो के रूप मे जगल, मवेशी, गैर इमारती लकडी वाले वन का उत्पादन, कृषि और चारागाह भूमि साम्मिलित हैं। इन स्थानो पर जीवन–यापन विधियो मे मवेशी मुख्य भूमिका निभाते हैं। ग्रामीण वर्ग मे, मुख्यत· उत्तराखण्ड क्षेत्र की महिलाओ को घर के अदर एव बाहर के कार्यो, जैसे पानी एकत्र करना, ईंधन हेतु दूर दूर से लकडी एव चारा लाने के कारण अत्याधिक बोझ सहन करना पडता है। चारे की प्राप्ति मे गुणवत्ता, मात्रा तथा तकनीकी बाधाऐ आती है। *थायोसैनोलीना मैक्सिमा* (राक्ज्ब) कुट्ज (झाडू घास) की बहुउपयोगिता एव उच्च पौष्टिक चारे के कारण ग्रामीण वर्ग द्वारा इसका घरेलू उत्पादन एक विकल्प के रूप मे महत्वपूर्ण है। इस घास को पौष्टिक चारे के रूप मे, जैविक स्क्रीन तथा गमले मे घरेलू पौधे के रूप मे, झाडू बनाने तथा दवा के पौधे के रूप मे, बढावा देने हेतु, नैनीताल एव आसपास के गॉवो मे नर्सरी के रूप मं स्थापित किया गया है। इस बहुउपयोगी घास का घरेलू उत्पादन उत्तराखण्ड की महिलाओ के कार्य बोझ एव आर्थिक दशा को सुदृढ करेगा। इसे परिचर्चित करने हेतु अनेक कार्यशालाऐ, जागरूकता कार्यक्रम ग्रामीणो हेतु चलाये गये है तथा प्रचार सामग्री का वितरण इस क्षेत्र के ग्रामीण वर्ग मे किया गया है।

सांकेतिक शब्द : *झाडू घास, मध्य हिमालय, चारा, घरेलू उद्योग*

Introduction

The major components of life-support system in the hilly areas are livestock, forests, agricultural and grazing lands. These components form an intricate web and livestock play a major role in sustaining the inter-linkages. Almost every family maintains some livestock, consisting of cows, oxen, goats and buffalo also. Besides contributing to a balanced diet and some income through the sale of extra produce, livestock supports crop production by providing much needed farm power and manure, vital for the prevailing organic agriculture in the hills. The support of agronomic production per ha of cropland requires the net production (in terms of green leaves for animal fodder, dry leaves for mulch, and wood for cooking) from 15 ha of well-stocked forest[1,2]. However, presently only 2 ha of forest is supporting 1 ha of agriculture in Uttarakhand.

Women and young girls of rural areas in the hills, who are responsible for fodder collection, in addition to water and fuelwood collection, have to carry these "necessities" as headloads for long distances and are the worst affected. The women are heavily engaged

in agricultural and non-agricultural activities for 7-8 hours per day throughout the year[3]. Various agricultural operations, *viz.*, field preparation, yield protection, yield enhancement and yield collection, account for about 60% of the total expended energy of women in the age group of 16-35 years and about 23% in animal care activities, such as cattle care during free-range grazing, stall feeding and in fodder and leaf litter collection[4].

The present work was carried out to explore the possibility of increasing the use of a multi-utility species, *Thysanolaena maxima* (Roxb.) Kuntze (broom grass), for reducing the drudgery of rural women in the hills by enhancing on-farm availability of fodder, and economic gains from the sale of brooms and other products prepared from this plant. The fodder obtained from *T. maxima* leaves is of good quality with digestibility at about 58%, crude protein in the range of 9.45%-18.07% and crude fibre at about 30%[5, 6, 7]. Further, the potential role of *T. maxima* in conserving soil and water under different land-use systems has also been recognized[8, 9].

Material and Methods

The study was carried out in the vicinity of Nainital town in Kumaun Himalaya with sites located between 343-2136 m amsl. Stock material (rhizomes) of *T. maxima* was obtained from plants growing in wild at Ghatgarh and Bhimtal areas in Uttarakhand.

Development of nurseries and transplantation: A mixture of farm yard manure (FYM), soil and sand (in the ratio of 1:1:1) was used for preparing nursery beds in the experimental sites at Bhimtal (1575 m amsl), Khurpatal (1627 m amsl) and Chilkiya (343 m amsl). The rhizomes were treated with a solution of Bavistin (1.5%, w/v) for four hours before plantation. The propagules were planted at a plant-to-plant distance of 24 cm. One year old plants raised in the nurseries were transplanted, with one plant m^{-2} density, at three different sites near the homes of voluntary farmers (Bhimtal, Khurpatal and Chilkiya) for field trials in the monsoon season of 2004, and also just before the onset of winter, later in the year.

Biomass removal experiments: These experiments included two different treatments. For these experiments, ten clumps were taken for each category of clipping.

i. In the first experiment, 15-months old plants were clipped twice at three different heights, first in October, 2004 and again in April, 2005.

   a. 20% of the total plant height

   b. 40% of the total plant height

   c. 60% of the total plant height

ii. In the second experiment, a certain proportion of culms were removed from the base of the plant, in October, 2004 and April, 2005,

   a. 25% of the total number of culms in the plant

   b. 50% of the total number of culms in the plant

The new growth that followed after treatments was harvested after six months and the data were recorded in terms of culm number, culm height, leaf/culm, belowground and total biomass, etc.

Drudgery reduction: The mean fresh weight of *T. maxima* leaf, mean number of leaves per plant and the number of plants per hectare were considered along with two levels of the harvest.

Income generation: For determining the income generated from the sale of brooms made out of *T. maxima* panicles, the cost of raw materials involved, the labour inputs and the market price of brooms were considered.

Awareness programmes: Information leaflets on the multiple uses and propagation of broom grass were developed and printed in Hindi and English for distribution among the common masses during orientation/ training workshops.

Results

Growth performance of *T. maxima* at three nurseries

The data recorded from 24 months old *T. maxima* plants indicated best growth performance at Khurpatal, followed by Chilkiya in terms of culm and leaf numbers, and culm height, while in terms of leaf area it was best at Bhimtal (Fig. 1). The soil analyses revealed higher organic matter and nitrogen contents at Khurpatal as compared to other sites. Judging by the highest leaf area attained by the plants, the nursery at Bhimtal could have resulted in best overall performance, had the plants not been damaged by the severe winter frost. The learning
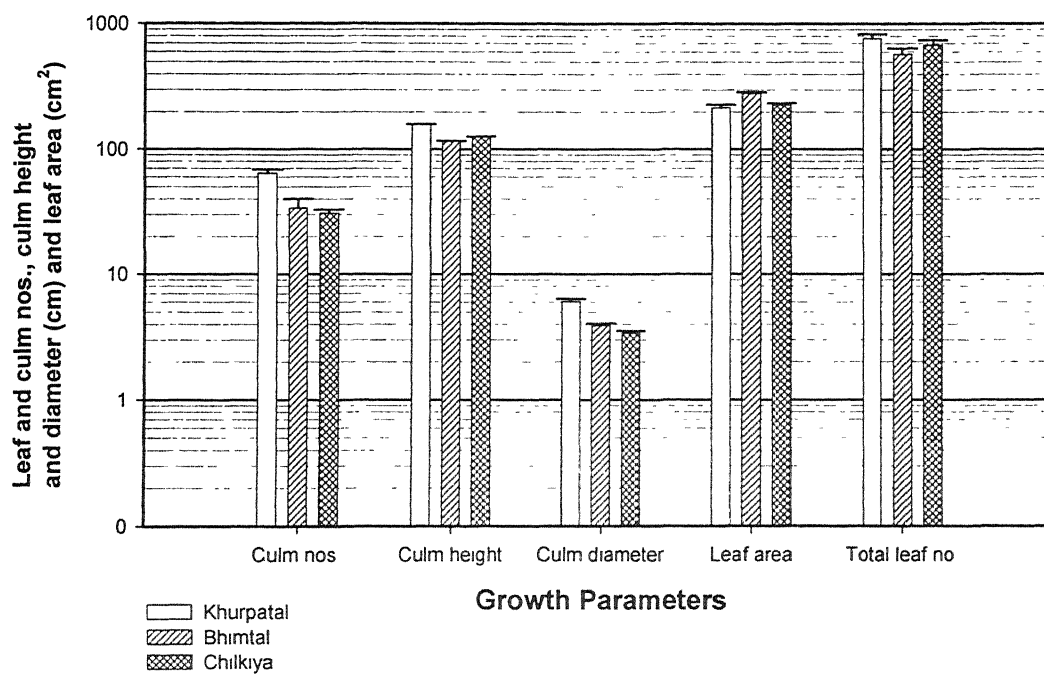
Fig. 1- Comparison of growth parameters of *T maxima* at three unprotected (open) experimental sites. The values represent mean of 10 plants. The values on y-axis are represented on $\log_{10}$ scale
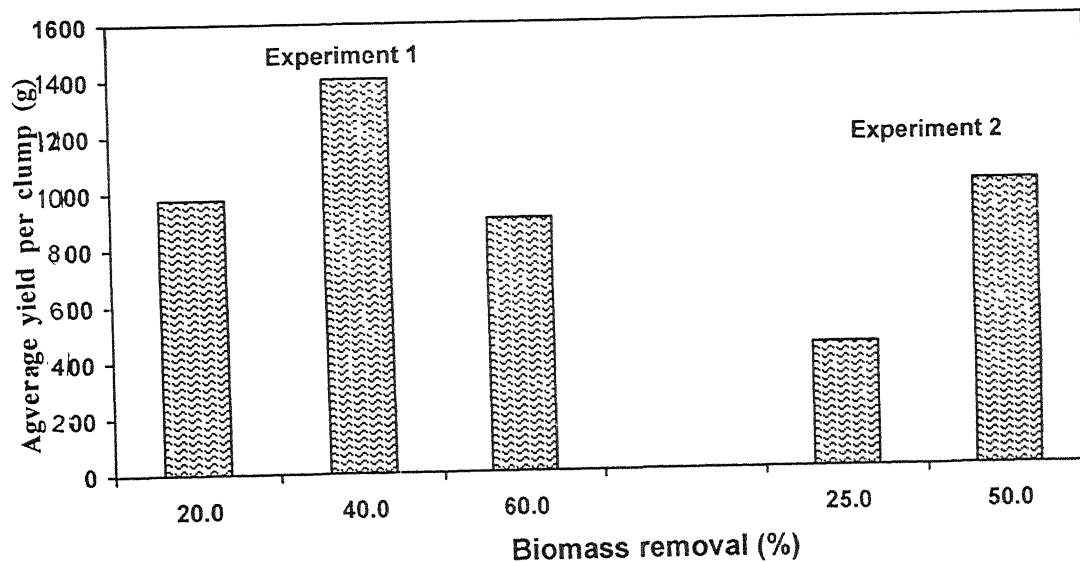
Fig. 2 - Comparison of yield per clump (g) from different clipping experiments. Data were recorded after 6 months.

lesson from this is to harvest above ground biomass well before the onset of winter frost.

Biomass removal experiments

ANOVA showed that there was significant ($P<0.05$) difference in the amount of leaves per plant (no. of leaves per culm x no. of culms) across different treatments and across the years of study ($P<0.01$). The first experiment showed that 40% clipping yielded best results, though yields were also reasonable with 60% clipping (Fig. 2). In the second experiment, 50% removal of culms yielded best results. Since among the treatments, 40% clipping gave the best results, this could be recommended as a practice when the plant is used as a source for green fodder.

From these experiments, the yield obtained at 40% and 60% level of harvest indicates that if broom grass is grown in one hectare of land, close to a village, it can save rural women up to 290 headloads (30 kg per headload as observed in the present study) of fodder collection per person in a year. This would provide sufficient fresh fodder (@20 kg per animal per day (as observed in the present study) for one milch animal for about 10 months in a year. The basic data for above calculations are summarised in Table 1.

Table 1 - Yield of fodder leaves from *T. maxima*

Parameter	Khurpatal	Chilkiya
Average number of leaves per plant	761.00	675.33
Average fresh weight of a leaf in g.	1.90	1.90
Average no. of fresh leaves per plant	1445.90	1283.13
Average weight of fresh leaves in kg	1.45	1.28
Fresh weight of leaves @ 40% harvest	0.578	0.513
Fresh weight of leaves @ 60% harvest	0.868	0.770
Average no. of plants per ha	10000	10000
Fresh weight of leaves in tonnes per ha @ 40% harvest	5.78	5.13
Fresh weight of leaves in tonnes per ha @ 60% harvest	8.68	7.70

Income Generation

The entrepreneur earning from the sale of brooms purchased *T. maxima* panicles from various people (who collected them from forests) at the rate of Rs. 350.00 per bundle that provides raw material for 90-100 brooms. The entrepreneur also purchased plastic sleeves for making the broom handles and a polythene bags for packing the prepared brooms from Moradabad (a town in U.P. about 100 km from the village). The total cost of production was about Rs. 7-8 per broom and it was sold in the Ramnagar market at a price of Rs. 15-18 depending upon its weight. This activity yielded the entrepreneur an additional income of Rs. 7000-10000 seasonally.

Awareness programmes

A number of visits were made to various sites for the collection of data and interaction with the villagers. 'Mahila Mangal Dal', 'Ekta Swayam Sahayata Sangh' and 'Sainiaon ka Sangh' were the major women self-help groups that were enthusiastic to adopt the cultivation of *T. maxima* in their villages. Workshops and meetings were held in villages located in the hilly and bhabar regions of Kumaun, Uttarakhand. In these workshops, the target audience were mainly women because introduction of *T. maxima* would be of most benefit to them in terms of reduction of drudgery in fodder collection and for income generation through the sale of brooms. During the workshop, techniques of broom grass propagation were demonstrated and some rhizomes/slips (planting material) of this multiuse plant were distributed to interested participants. In addition to this, leaflets prepared for the project were distributed to women and other participants for reference.

Discussion

Thysanolaena maxima (Roxb.) Kuntze is being used in bioengineering works in some parts of Central Himalaya through planting of rhizome raised plants in diagonal rows[8, 9]. Its main function in bioengineering is to shield, catch and to provide reinforcement to the sliding soil[5, 10] . The grass is also grown as an ornamental potted plant as well as an effective biological screen in the gardens. This plant is also known for its medicinal uses. Decoction of roots is used as a mouthwash during fever. A paste of dried or fresh roots is applied on the skin to check boils[11]. The traditional healers of Bastar region use the decoction of whole herb internally for many purposes. It is used by healers as a remedy in the treatment of high fever[12]. The traditional healers of Pendra region suggest the patients having the problem of bavasir (piles) to wash the anus with this decoction[13].

During the present project, work of an entrepreneur in Village, Sawaldeh of Block Ramnagar, District Nainital (Uttarakhand) who runs an enterprise based on the production and sale of brooms was also studied. It was found that his venture yields earnings of about Rs. 7000-10000 per year. The brooms are in much demand in the urban and semi-urban areas of Uttarakhand and are at present bought from Uttar Pradesh to be sold by the retail shopkeepers in the region. Thus, there is considerable potential of *T. maxima* as an income generating activity, based on the sale/making of brooms/ potted plants, etc. for rural women folk of Uttarakhand. The results of the clipping experiments clearly indicate that this grass yields good quality green fodder for milch animals, especially during the lean period.

Thus, this multiuse species, *T. maxima,* has the potential of providing supplemental income to the womenfolk of Uttarakhand besides reducing their workload in terms of fodder collection. The following income generating activities need to be promoted: (i) sale of brooms made from this grass, (ii) sale of surplus milk due to increased production by feeding good quality of fodder to the milch animals, (iii) sale of (a) potted plants for decorative purposes, and (b) slips for creating biological screens, (iv) sale of propagules for use by other farmers, and (v) sale of various components for use in traditional medicines.

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Environmentally affected allelopathic response of a few weeds on late blight disease of potato

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Abstract

The course of development of late Blight disease of potato was studied in experimental plots planted with seed tubers of cultivar Khasigaro, treated with extracts of weed flora namely, *Ludwigia purviflora* Roxb. *Polygonum plabejum* R. Br. and *Anisomeles ovata* Br During two crop seasons 2004-05 and 2005-06. maximum infection of plants occurred during rainy days with moderately low temperature and high humidity prevailing within the plant canopy in both treated and untreated plants. The incidence of late Blight was found to be significantly delayed and percent Blight intensity sufficiently decreased when the plants were treated with extracts of *Ludwigia* and *Anisomeles* Complete eradiction of the disease in the plants treated with the weed extracts was, however, not possible. Inhibition of growth of *Phytophthora infestans* was also evident with the weed extracts during *in-vitro* studies.

Key words : potato crop, allelopathic response, weeds, environmental factors.

सारांश

आलू में 'लेट ब्लाइट रोग' के विकास की गति का अध्ययन 2004-05 तथा 2005-06 के उपज काल मे कुछ प्रयोगात्मक भूखण्डो मे 'खासीगारो' नस्ल के बीजकदो को उगाने के बाद, *लडवीजिया पार्वीफ्लोरा* राक्सब, *पालीगोनियम प्लेबेजम* आर बीआर तथा *एनीसोमेलिस ओवेटा* ब्रा जैसे खरपतवारो के सार तत्व द्वारा प्रतिपादित कर के, किया गया। उपचारित तथा अनुपचारित दोनो प्रकार के पौधो मे अत्यधिक सक्रमकता बरसात के दिनो मे दिखी, जब तापमान सतुलित रूप मे कम तथा आद्रता अधिक थी। ऐसी परिस्थिति मे जब पौधो को *लाइवीजिया* तथा *एनीसोमेलिस* के सारतत्व द्वारा प्रतिपादित कर दिया गया, 'लेट ब्लाइट रोग' का आपतन सार्थक रूप मे विलम्बित दिखा, साथ ही साथ ब्लाइट की तीव्रता का प्रतिशत भी पर्याप्त रूप मे कम था। खरपतवार के सार तत्व द्वारा प्रतिपादित पौधो मे इस रोग का पूर्ण निस्तारण सभावित नही था। ऐसे पौधो के अत पात्र अध्ययन द्वारा *फाइटोप्थोरा इनफेस्टेन्स* वृद्धि का निषेध सुस्पष्ट था।

सांकेतिक शब्द : *आलू की उपज, एलीलोपैथिक प्रतिक्रिया, खरपतवार, पर्यावर्णीय कारक*

Introduction

The potato crop is affected by several fungal diseases causing heavy loss in the yield. The late blight alone causes 70-80 percent loss in potato crop[1]. The influence of environmental factors on the development of late blight disease has been emphasized by many workers. Couzens and Evans[2] stated that under appropriate climatic conditions, infected tubers give rise to initial foci of infection, the dimension, frequency and persistence of which vary from season to season and from place to place. Control of late blight disease has been a major issue before the workers, as till date full eradication of the pathogen by the fungicides, has not been confirmed. Now a days, in view of the toxicity caused by the chemicals during cultivation and storage, eco-friendly strategies, such as use of plant products, parasitoids, predators and pathogens[3] are being adopted. Certain metabolites of several higher plants are known to produce toxic effects against spore germination and mycelial growth of phytopathogenic fungi[4]. The present study aims at investigating the pattern of incidence and development of late blight disease of potato crop as affected by climatic conditions of Assam using tubers treated with some common weed plant extracts available in the potato cultivated areas.

Material and Methods

1. The study was made in four thoroughly ploughed experimental plots, measuring 5m long and 4m wide, making three replications. Healthy potato seed tubers were hand planted in the base of ridges of rows 70cm apart with 20cm spacing in between the tubers. A total of 120 seed tubers of average size of potato cultivar 'Khasigaro' were planted in each plot. After the plants attained 7cm height, earthing was done. The plants were examined from time to

time for the appearance of late blight by visual estimation[5]. A few seed tubers which did not germinate were discarded.

2. Relative humidity, temperature and rainfall data were recorded during the entire period of investigation.

3. Root, leaf and stem extracts from three fern plants namely, *Ludwigea purviflora* Roxb., *Polygonum plebejum* R.Br. and *Anisomeles ovata* Br. were prepared by the method followed by Parihar and Bohra[6]. The above plant species were selected because they grow profusely in the potato cultivation areas of Assam and also utilized by the native tribes for medicinal purposes. 5 g of fresh plant parts were washed 2-3 times with tap water and then surface sterilized with 90% alcohol. Subsequently, the material was grounded in 50ml acetone. The acetone macerates were kept for 24h at room temperature, to let the acetone evaporate. In the remaining residue, 50ml of distilled water was added. Macerates were squeezed through double layered muslin cloth and filtered through filter paper. After filtration, aliquots were centrifuged at 10,000 rpm for 20 min. The supernatant was filtered through Whatman No. 1 filter paper and then sterilized by passing through 0.2 micron disposable filters. The extracts (10%) thus obtained were used for the studies. Aquous plant parts extracts were simultaneously prepared by using distilled water.

4. The antifungal activity of the plant extract on the mycelial growth of *Phytophthora infestans* was measured by adding 1ml extract to 10ml 'Limabean Agar media' (Savage *et al.*[7]). A small portion of sporulating colony was placed in the center of the petridishes containing the culture media. Diameter of mycelial mats were measured after 72 hours. The rate of spore germination was evaluated by employing spore germination tests in cavity slides (Chandrol and Karkum[8]). Spores were collected by gently brushing the surface of infected leaf, with a sterile needle, suspended in distilled water. For each treatment, one drop of extract with loopful of spore suspension was put in cavity slides and covered with cover slip. Suspensions prepared in a similar manner in sterile water served as control. After incubation for 12 hours at 18°C, at least six microscopic fields were examined to obtain germination percentage by using the following formula.

$$\text{Percentage of spore germination} = \frac{\text{Total number of germinated spores} \times 100}{\text{No of spores/loop in spore suspension}}$$

Results and Discussion

The antifungal property of the extracts of the test plants, studied as a part of the present experiment revealed significant influence of the extracts on the mycelial growth and sporulation of the causal organism of late blight disease *Phytophthora infestans* A significant reduction in mycelial growth was noticed when treated with acetone extract of *Ludwigea purviflora* (Roxb.)

Table 1 - Effect of plant extract on mycelial growth (mm) and germination of sporangia (%) of *P. infestans*.

Plant extract		Control		Root		Stem		Leaf	
		A	B	A	B	A	B	A	B
		18.4	78.2	-	-	-	-	-	-
T_1	Aquous	-	-	08.3	20.6	12.3	33.4	10.2	24.4
	Acetone	-	-	05.3	14.5	09.6	32.4	04.3	21.6
T_2	Aquous	-	-	12 6	34.6	16.5	57.8	15.4	47.8
	Acetone	-	-	13.0	47.2	12.4	49.6	15.9	53.2
T_3	Aquous	-	-	09.2	32.3	05.4	57.8	09.3	27.4
	Acetone	-	-	06.3	39.3	17.9	73.2	07.5	19.3

C.D. at 5% T_1=0.03, T_2= 0.06, T_3= 0.03

T_1 = *Ludwigia purviflora*; T_2= *Polygonum plebejum*; T_3= *Anisomeles ovata*

A= Mycelial growth; B = Germination of sporangia

Table 2 - Course of development of late blight disease in plants treated with weed plant extract.

Treatment	No. of tubers		Days of observation											
			2004-2005						2005-2006					
			17th Dec	24th Dec	31st Dec	7th Jan	14th Jan	21st Jan	28th Dec	4th Jan	11th Jan	18th Jan	25th Jan	1st Feb
Control	118	I	0.09	00.42	03 02	12.43	78.16	03 12	06.3	0 75	29.53	68 0	86.5	37 0
		II	-	01 85	12.44	23.54	53 24	15 33	0 3	02.43	24.30	46 74	65.00	12.44
		III	-	06.37	35 42	74.56	83.24	94.72	-	02.33	26.33	57.84	76 42	90.00
		IV	N S.	09.46	23.74	64.75	86 45	100.0	N S	05.40	18.76	68.32	89 00	97. 06
Extract I	116	I	-	-	01 79	06.42	32.68	54.62	-	04.32	06.33	42.67	48 73	66.33
		II	-	-	-	04.87	38.75	22.54	-	02.33	02.76	12.43	56 42	34.00
		III	-	-	-	10.72	36.64	46.08	01 67	02.33	06.00	18.52	67.86	74.34
		IV	N.S.	N.S.	N.S.	04.85	34.42	43.45	N S	02 00	05 32	12 53	45 89	57.00
Extract 2	115	I	0 14	02.28	05.40	26 04	82.06	26.54	02.12	02.56	04.36	37.65	59.00	32.02
		II	-	06.76	24.63	63.22	67.00	28.40	-	-	02.34	36.42	62.65	72.43
		III	-	22.43	42.86	72.35	78.44	96.37	-	-	01.65	48.92	72.54	81.00
		IV	N.S.	08.97	28.76	71.23	84 25	100 0	-	-	03.65	53.87	62.00	71.90
Extract 3	112	I	-	-	04.72	26.04	40.86	44.58	-	01.42	12.43	14.67	28.75	48 76
		II	-	-	-	08.93	12.33	16.52	-	-	05.42	13 00	15 65	42 64
		III	-	-	-	24.53	37.84	63.94	-	-	05.56	32 53	38 77	56.53
		IV	N.S.	N.S.	N.S.	25.46	41.23	62.56	-	-	03.42	16.78	29.65	43.12
Total weekly rainfall (mm)			00.65	17.80	10.50	14.35	18.76	08.36	08.73	12.60	16 52	22.64	18 52	12.00
Av weekly humidity (%)			73.55	77.76	85.78	89.10	86.60	83.44	83.56	78.24	89 00	82.14	80 17	67 84
Av. Weekly temperature (^{0}C)			19.35	18.58	20.36	18.76	20.08	20.64	16.24	19.00	16 00	20.46	17.64	22.38

C.D. for treatment at 5% = 0.043

I = Percentage of plants bearing lesion on leaves only

II = Percentage of plants bearing lesions on stem only

III = Percentage of plants bearing lesions on both Stem and leaves

IV = Percent blight intensity

Control = without plant extract

Extract 1 = *Ludwigea purviflora*

Extract 2 = *Polygonum plebejum*

Extract 3 = *Anisomeles ovata*

NS = *Not significant*

and *Anisomeles ovata* (Br.). Among these two, percent inhibition was more in *Ludwigea* as compared to the other two extracts. The effect of *Polygonum plabejum* (R.Br.) was less as compared to the extracts of other two plants. However, it has been revealed that all the three extracts showed inhibition of the fungus in cavity slide tests. Percent germination of sporangia was reduced in all the extracts as compared to control. Maximum

Table 3 - No. of tubers/plant, weight and yield in controlled and treated plots.

Treatment	No.of plants/plot	No. of tubers/plant (average)		Weight/tuber(g) (average)		Yield/kg seed tuber(kg)	
		2004-05	2005-06	2004-05	2005-06	2004-05	2005-06
Control	118	10 64	12 33	32 76	39.65	04 24	09 32
T_1	116	15.43	18.87	74 06	56.33	19.62	13.53
T_2	115	07.52	19.12	24.45	44.00	05.59	10.33
T_3	112	14.87	24.53	73.76	63.00	16 87	12.42

C.D. at 5% =0.572

Control = Without plant extract

T_1 = *Ludwigea purviflora*

T_2 = *Polygonum plebejum*

T_3 = *Anisomeles ovata*

inhibition in sporangial germination was noticed in the slides treated with root extract of *Ludwigea* (Table 1).

From the study of the course of development of late blight in potato crop in both the controlled and treated plots, it is seen that once the disease appeared in the plants, the process of infection continued till most of the plants get infected. Closer inspection of the first appearance of blight infection reveals that initial infection occurs at the terminal leaflets of the plants, and gradually the disease migrates to the lower leaves and stems, which suggest emergence of the disease from an initial airborne inoculum coming probably from other infected plots.

In the plots treated with extracts of *Ludwigea purviflora* (Roxb.) and *Anisomeles ovata* (Br.), a significant delay in the emergence of the disease (Table 2) was observed in both consecutive crop seasons. The percent blight intensity in the plots treated with these extracts was sufficiently low as compared to other plots. The quantity of tubers per plant, the average size as well as yield has been significantly higher in the plots treated with these weed extracts (Table 3). However, the same has not been noticed with with extracts of *Polygonum plabejum* (R. Br.) during observations made in 2004-05. Initial infection in leaves occurred on 17th and 28th December in the two crop seasons respectively in the plots without any treatment and in the plot treated with *Polygonum plebejum* (R. Br.), the infection occured when the plants were 46 and 58 days old. The infection gradually spread to other plants in the vicinity and within a period of 5 weeks, maximum number of plants bore lesions of blight.

Rainfall probably plays a significant role in the emergence of late blight. During the entire period of investigation in the two consecutive crop seasons, it was revealed that initial infection of late blight occurred on the day only when there was a mild rainfall resulting in increased humidity and a temperature conducive for disease development. Spread of lesions also were more frequent during rainy days and cloudy weather.

Late blight disease of potato is by far the most serious disease of potato in Assam. It causes significant damage to the crop every year despite sincere efforts made by researchers and agricultural experts throughout the state. The environmental factors which prevail during the potato growing season, most probably, become suitable for the emergence and outbreak of the disease as has also been seen in the present investigation.

Every growing plant produces compounds, known as allelochemicals, which show either stimulatory or inhibitory effect on other plants[9]. The antifungal as well as stimulatory or inhibitory effect of a number of higher plants against crop plants and pathogens have been reported[10]. Phukan[11] obtained inhibition of growth of *Phytophthora infestans* by using extracts of fern plants such as *Adientum reniforme, Dryopteris filix mas* and *Polypodium decorum.* Bohra and Purohit[12] obtained inhibition of *Aspergillus flavus* by using extracts of *Azadirachta indica.*

Conclusion

Management of plant diseases with measures other than use of fungicides has been drawing attention since

last three decades, because of severe ill effects of the chemicals on the overall health of both the producers and consumers. Chemical pollution of the environment as a result of uncontrolled applications of these compounds has been creating a serious problem to everyone's livelihood. The toxic effects of agricultural and other commodities treated with chemicals and preservatives are well known. Hence, workers all over the world, at present, are in search of some alternative measures, free from such problems, which may be exploited for the betterment of the society as well as increased productivity of essential commodities. Allelopathy in plant disease control, if generated properly and extensively, may prove to be one of the suitable alternatives in management and increased productivity of crops.

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